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ATHEROSCLEROSIS IN THE LA/N-CP RAT - THE PRIMARY ROLE OF SUBENDOTHELIAL MIGRATION OF INJURED ENDOTHELIAL CELLS

BY

OWEN ROBERT HEISLER

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

EXPERIMENTAL SURGERY

DEPARTMENT OF SURGERY

EDMONTON, ALBERTA

SPRING 1987

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ISBN 0-315-37754-2

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TITLE OF THESIS: LA/N-cp Rats - An Atherosclerosis Model

DEGREE: Master of Science

YEAR THIS DEGREE GRANTED: 1987

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ATHEROSCLEROSIS IN THE LA/N-CP RAT - THE PRIMARY ROLE OF SUBENDOTHELIAL MIGRATION OF INJURED ENDOTHELIAL CELLS

submitted by Owen Robert Heisler

in partial fulfillment of the requirements for the degree of Master of Science in Experimental Surgery.

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Date: 21 April 1987

ABSTRACT

ATHEROSCLEROSIS IN THE LA/N-CP RAT - THE PRIMARY ROLE OF SUBENDOTHELIAL MIGRATION OF INJURED ENDOTHELIAL CELLS

Atherosclerosis research has long been hampered by lack of an inexpensive, rapidly breeding animal model which develops lesions similar to those of man within a short experimental time frame. The genetically obese, hyperlipidemic LA/N-cp rat may provide such a model.

Four corpulent and 4 control thin male LA/N-cp rats at both 6 and 14 months of age were perfusion fixed. The aortic arch was removed and divided into equal halves. One half was prepared and examined by scanning electron microscopy (SEM). The other half was imbedded into epon blocks from which thick (2 micron) sections were prepared and examined by light microscopy and thin (60 - 90 nm) sections prepared and examined by transmission electron microscopy (TEM).

Lesions were present in both control and corpulent rat aortas, occurring earlier and with more severity in corpulent and aged rats. Areas of endothelial irregularity and denudation plus debris laden vacuoles were present in endothelial cells. Macrophages attached to the surface and penetrating into the subendothelium were identified by TEM in old lesions. Smooth muscle cells, lipid and amorphous material appeared in the subendothelial space in a gradated fashion in the rats. Abnormal endothelial cells which had been overlapped by adjacent endothelial cells appeared in apparently earlier lesions.

The LA/N-cp rat, an ever more promising model, shows a wide variety

of aortic lesions on a normal rat chow diet. Lesion incidence and severity increases with age and the corpulent genotype. The response to injury hypothesis proposes that the subendothelial migration of macrophages is an early event of atherogenesis. Evidence that this occurs late and that subendothelial migration of damaged endothelial cells is an inciting event in atherosclerosis has been identified.

ACKNOWLEDGEMENT

I would like to acknowledge the excellent technical assistance provided by Mrs. Edith Schwaldt in not only the use of the electron microscopes and specimen preparation but also in photography.

I would also like to acknowledge the assistance of Miss Tamie Heisler in preparation of the manuscript.

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ATHEROSCLEROSIS

Introduction

Atherosclerosis has been defined in Dorland's medical dictionary as "a form of arteriosclerosis in which atheromas containing cholesterol, lipoid material and lipophages are formed within the intima and inner media of large and medium sized arteries"[1]. The vague, descriptive nature of this definition reflects the inadequate understanding of this serious disease which continues to exist in the 1980's. Extensive investigation has been done and data on multiple different aspects of atherosclerosis continue to accumulate at a rapid pace. However, Mother Nature has still not parted with the secret of the etiology of atherosclerosis. The deficiency of a cheap, easily studied and representative animal model remains a formidable stumbling block in the search for not only a cause but also a cure for this, the greatest cause of premature human death in Western society. The raison d'être for this investigation revolves around the continuing search for this elusive animal model. The purpose of this study is to both characterize and make an evaluation of a potentially good rodent model of atherosclerosis.

Prior to detailing the design and findings of this investigation, current concepts of the epidemiology, pathology and pathogenesis of atherosclerosis will be examined followed by an overview of current animal models of atherosclerosis. With this as a background, the

literature on the animal model I have evaluated will be examined. An outline of current deficiencies in the atherosclerosis data base along with a working hypothesis concludes this preliminary review.

Epidemiology of Atherosclerosis

Atherosclerosis, by current standards, is more a pathologic entity than a clinical disease. It is entirely possible and unfortunately very often the case that an individual will have very severe atherosclerotic disease and remain asymptomatic[2]. In order to ascertain some sense of the incidence and prevelance of the disease it is necessary to examine the incidence and prevelance of the clinical complications of the disease. The most useful clinical indicator is ischemic (coronary) heart disease, as virtually all persons who are symptomatic with ischemic heart disease have coronary atherosclerosis[2]. A substantial proportion of individuals who suffer strokes have atherosclerotic disease, though this is not quite as reliable an indicator as ischemic heart disease since a small percentage of strokes occur as a result of cerebral hemorrhage or thrombosis. Other manifestations of atherosclerosis include aortic aneurysms, ischemic renal disease and peripheral vascular disease which on occasion will lead to gangrene of the extremities with the attendant possibility of amputation. Without a doubt, atherosclerosis is the leading cause of mortality and morbidity in industrialized countries. In the 1980's ischemic heart disease, only one of the mainifestations of atherosclerosis, by itself continues to be the leading cause of death in males over age thirty-five and all persons over age forty-five

in the United States[2].

Considerable research has been done in an attempt to elucidate the risk factors for atherosclerosis. The best study to date as well as the most publicized in both the medical literature and lay press was and is being done in Framingham, Massachusetts. Castelli and associates have selected inhabitants of this community and followed them prospectively over the last twenty years. Multiple publications have resulted from this investigation documenting the devestating effect atherosclerosis has on a community as well as evaluating many different risk factors. By age sixty, one in five males and one in seventeen females in the study group had some form of coronary heart disease[3 - 6]. As well, one in fifteen males and females eventually developed a stroke. Factors found to have a significant association with atherosclerosis include an elevated total to HDL serum cholesterol, increased blood pressure, cigarette smoking, obesity, increased blood sugar, lack of exercise, stress and EKG changes.

By far and away the most important risk factors are cigarette smoking, hypertension and hyperlipidemia[7]. Cigarette smoking has a very strong relationship with atherosclerosis, though fortunately this risk can be significantly decreased by stopping smoking[6,8 - 10]. A consistently elevated blood pressure, especially when above 115 mm Hg diastolic, has a strong correlation with atherosclerotic diseases[11 - 13]. Current research on the effects of hyperlipidemia suggests that the development of atherosclerosis in an individual is related more to an elevated level of serum cholesterol than an elevated level of triglycerides[14]. Furthermore, although cholesterol present in low density lipoproteins (LDL) is positively associated with coronary

disease, cholesterol present in high density lipoproteins (HDL) has an inverse correlation. Very low density lipoproteins (VLDL) seem to be related more to triglyceride levels than serum cholesterol and as such do not appear to be a strong independent risk factor. The reason the male to female ratio for atherosclerosis is so strongly tipped toward the male side prior to the female menopause, with a rapid shift towards unity afterward, may be variations in the LDL and HDL levels secondary to sex hormones. The association of other apparent risk factors such as obesity, lack of exercise, and alcohol consumption with atherosclerosis may also be a reflection of alterations in the HDL and LDL levels[15].

Increasing age directly correlates with increasing risk of atherosclerosis. Other irreversable risk factors include genetically inherited traits, either in the form of a disease such as familial hypercholesterolemia or as a general genetic predisposition for the development of atherosclerosis. Evidence abounds implicating diabetes as a significant risk factor, though studies are equivocal on the effects of borderline hyperglycemia[16 - 18]. Fish oils and aspirin, both of which inhibit platelet aggregation, seem to have a favorable effect on lipoprotein levels which has lead to a great deal of research on the relationship between thrombotic tendencies and lipids[19,20]. There is no agreement on the often cited relationship between Type A personality and coronary artery disease - it appears that there is only a relationship insofar as the Type A people are more likely to report their symptoms[21 - 23].

Pathology of Atherosclerosis

In considering the pathology of atherosclerosis we must examine two lesions - the fatty streak and the atheromatous plaque[24 - 28]. It remains controversial whether or not the fatty streak is a precursor to the atheromatous plaque and both sides of this argument will be presented llowing a brief description of the two lesions.

The Fatty Streak

Grossly, fatty streaks are flat, narrow, elongated yellow lesions which evolve from multiple yellow spots in the blood vessels. They are usually quite small and may require special staining (for example with Sudan IV) to be seen. The lesions are ubiquitous and appear in all children regardless of geography, race, ~~sex~~ or environment, possibly at birth and definitely by one year of age. Lesions first appear in the aorta and later in the coronary and cerebral arteries. Early in life, the lesions are localized to the thoracic aorta, particularly in the aortic valve ring region and the area of the ductus arteriosus scar. They also occur near the ostia of aortic branches. Approximately ten percent of the aortic surface is covered with the lesions in the first decade of life, progressing to thirty to fifty percent of the surface by the third decade. Following this, they begin to decline in prevelance. In the coronary arteries the lesions occur mainly in the proximal segment of the left coronary artery[29].

Histologically, fatty streaks are characterized by lipid deposition in the intima, both as intracytoplasmic lipid in smooth muscle cells and/or macrophages (so called foam cells) as well as extracellular collections[30]. There is some debate as to whether the foam cells

arise from within the intima or whether they are a blood derived monocyte-macrophage[31,32]. Variable amounts of proteoglycans, collagen and elastic fibers are also present within these lesions though the amount varies from lesion to lesion.

Atheromatous Plaques

The atheromatous plaque, also referred to as the fibrous, fibrofatty, lipid or fibrolipid plaque, is the hallmark of atherosclerosis. Grossly, these lesions are white to whitish yellow and protrude into the lumen of the artery, ranging from 0.3 to 1.5 centimeters in diameter though they may coalesce to form larger masses. On sectioning, they may contain a yellow gruellike fluid (thus the derivation of atheroma - the Greek word for gruel).

The distribution of the plaques is quite uniform and different from the distribution of fatty streaks[33]. The abdominal aorta is most prominently involved, rather than the thoracic aorta, especially near the ostia of the major branches. However, the coronary arteries are involved in their proximal portions, as is the case with fatty streaks.

Histologically, the plaques are characterized by smooth muscle cell proliferation, accumulation of connective tissue fibers and lipid deposition[34]. The plaques are composed of a fibrous cap of smooth muscle cells in a lacunar like arrangement in which lacunae have alternating layers of basement membrane and proteoglycan. Beneath this are highly cellular areas with smooth muscle cells and macrophages which may themselves contain lipid droplets[35]. Cell distortion secondary to the lipid accumulation has made it difficult to determine what proportion of an advanced lesion is composed of macrophages and what portion is made up of smooth muscle cells. Current studies

involve use of monoclonal antibodies to quantify the proportions of smooth muscle cells and macrophages in various lesions, although the antibodies used are still quite nonspecific[36-38]. As observed grossly, the smooth muscle-connective tissue arrangement in more advanced lesions often contains a necrotic, lipid rich gruel core. The gruel is composed of necrotic debris, cholesterol crystals and calcium. The amount of lipid and fibrous tissue varies between lesions and locations; for example, coronary artery lesions are often largely fibrous in comparison to the more lipid filled aortic lesions.

Various changes often occur within the fully developed atheromatous lesions. The lesions may ulcerate which can lead to embolization of material (eg. cholesterol emboli). The emboli have the potential to occlude smaller vessels downstream as happens in the case of cerebral strokes following embolization from the carotid artery. Alternatively, the surface ulceration may lead to a superimposed thrombosis which narrows the lumen and ultimately completely occludes it. In the case of the coronary arteries this leads to the symptoms of ischemic heart disease. With time, atheromatous plaques tend to become vascularized. Intraplaque hemorrhage is a feared complication which may result in sudden, total luminal occlusion. This appears to be a common etiology of coronary occlusion and resultant myocardial infarction or sudden death in previously asymptomatic people. With time, the progressive destructive type of lesions can lead to a weakening of the arterial wall and aneurysm formation with possible eventual rupture as occurs in aortic aneurysms. On the other hand, the progressive cellular type of lesions may become calcified leading to "lead pipe" type arteries as is sometimes seen in the peripheral arteries.

Beget or Begone?

As stated, the fatty streak is ubiquitous. A great deal of controversy exists as to whether the fatty streak progresses and later develops into the atheromatous plaque or whether it is an entirely separate phenomenon with no relationship whatsoever with separately developing plaques. It would intuitively seem that there is a relationship, as both lesions involve the intima, both are characterized by lipid deposition and both exhibit smooth muscle cells and foam cells within the lesions. There is some experimental evidence for a relationship based upon changes seen in injured vessels but the evidence is not strong[39].

There are various chemical differences between fatty streaks and atheromatous plaques, especially in their fatty acid, lipid and fibrinogen content[40]. Although the lipid in both the atheromatous plaque and fatty streak is mainly cholesterol ester, oleic acid is the principle esterified fatty acid in the fatty streak whereas linoleic acid is the principle esterified fatty acid in the atheromatous plaque.

Examination of coronary arteries has provided the most quoted evidence for a product-precursor hypothesis[41,42]. Much of this work has been done by Stary who has demonstrated that fatty streaks occur in similar locations to atheromatous plaques in the coronary arteries of young children[43]. McGill has presented evidence, again based on the examination of coronary arteries, that there is increased surface involvement in fatty streaks prior to later development into raised lesions[44]. However, the distribution of fatty streaks and atheromatous lesions do not mirror each other in the aorta, the most common site of atheromatous lesions, as they do in the coronary

arteries. In the aorta, the fatty streaks occur predominately in the thoracic aorta whereas atheromatous plaques predominate in the abdominal aorta. There is no associated increase in the incidence of fatty streaks in areas of the aorta in which atheromatous plaques are identified[45]. As well, as stated previously, the coronary lesions are much more fibrous than aortic lesions which may or may not indicate a different pathogenetic mechanism.

However, the controversy does not end at the precursor-product argument. If one accepts the theory that fatty streaks are indeed the precursors to atheromatous plaques, the question still remains as to whether there is a lesion which antedates the fatty streak and whether the fatty streak will regress either spontaneously or in response to different manipulations[46-50]. The role of diffuse intimal thickening, a separate lesion occuring at bifurcations which is similar to an atheromatous plaque except for the absence of any lipid, has not been clarified. Nor has it been determined how separate gelatinous lesions which contain large amounts of LDL and fibrinogen but little free cholesterol relate to the other lesions[51-53]. Some consider these lesions separate entities, attributing the intimal thickening to hemodynamic stress while others feel they are a stage in the development of atherosclerosis.

Pathogenesis of Atherosclerosis

With any disease, especially one as prevalent as atherosclerosis, the central aim of any research is to characterize the cause or etiology of the disease and trace the events in the development of the

disease (pathogenesis). If we know why and how a disease occurs, we can plan and devise strategies to either eliminate the cause (affect etiologic agents) or arrest the process at some stage of its development (affect the pathogenesis). Multiple theories have been formulated which attempt to assimilate and explain all the manifestations of atherosclerosis. None have yet satisfactorily unraveled the riddle of atherosclerosis[24-27,54-58]. This deficiency has resulted from the inability to compartmentalize neatly all the multifactorial aspects of the disease process. All theories attempt to account for the presence of cholesterol and smooth muscle cell proliferation in the lesions. The observed epidemiologic associations, especially the role of the major risk factors (smoking, hypertension and hyperlipidemia), is also usually considered although the tendency is to concentrate on only one of the factors, most commonly the hyperlipidemia. Each of the theories concentrates on the aspect(s) of the disease which it explains best. It is worthwhile to briefly consider each theory, not only to gain a better understanding of atherosclerosis but also to get a feel for the difficulty in defining this disease.

Until recently, there were only two major theories or variations thereof, the thrombogenic theory and the inflammatory theory. These two theories will be discussed first followed by a brief description of several other recently postulated hypotheses. A more detailed examination of the hypothesis which is currently in vogue and to date has been most effective in explaining the manifestations of atherosclerosis, the response to injury hypothesis, will conclude the discussion.

Thrombogenic (Encrustation) Theory

This is the oldest theory which was initially put forth by Rokitansky in the late nineteenth century[59]. The initial contention was that an abnormality of the blood exists which results in the deposition of fibrinous substances onto the arterial surface. As the theory maintained that the lesions were blood derived, it followed that the mass was thus composed of degenerated blood products. The observed lesions were felt to be blood proteins, cholesterol crystals and fatty globules which had been laid down on the surface and then continued to degenerate[60,61]. Mallory extended this concept somewhat when he noted fibrin-like material in the plaques which he attributed to organizing fibrin from the blood[62]. The appearance of the laminated nature of the atherosclerotic plaque was attributed by Clark to repeated deposition of blood elements prior to complete organization of a lesion which had already been deposited on the surface[63].

The thrombogenic theory fell into disregard for some time corresponding with interest in the inflammatory theory until it was revived and extended by Duguid in the late 1940's[64-67]. Duguid argued that if the fibrous reaction in the atheromatous plaque was due to reactive fibrosis (as proposed by the inflammatory theory) the arterial lumen should dilate rather than narrow as is observed to occur. Duguid supported this position with his observation that thrombi were present on microscopic examination of aortas even in young people. It was postulated that since fibrin is continuously formed and lysed it is only a small defect in this mechanism which is responsible for the formation of atherosclerotic lesions. This stimulated intensive investigation into possible thrombogenic factors which as an

offshoot led to a considerable data base on fibrinolytic-thrombotic systems[68-73].

The thrombogenic theory has often been criticized for not incorporating an explanation for the role of the assumed precursor lesions, the fatty streaks. In keeping with this theory, the fatty streaks may not be precursor lesions of the atheromatous plaques, as has already been discussed. The role which damage to the artery itself might play and the effect of hemodynamic factors such as elevated blood pressure were not accounted for in earlier versions of this theory. Advocates of the theory have incorporated explanations for these observations by postulating that the deposition of the debris from the blood is preceded by some form of endothelial damage which acts as an inciting factor[71].

The observation that mural thrombi are practically always on the surface of a preexisting atherosclerotic plaque and practically never on a normal arterial surface would appear to be in direct contradiction to the thrombotic theory[70].

Imbibition Theory

This theory, also known as the inflammatory theory, was initially proposed by Virchow who opposed Rokitansky's view on the grounds that subendothelial lesions could not be derived from surface deposits[74]. He proposed that there is an "irritation" of the intima by mechanical forces which leads to a loosening of the intimal structure and subsequent infiltration of plasma into the subendothelial space. The plasma components cause an inflammatory process which leads to formation of the atheroma from degenerated tissue. In response to these degenerated products, the intimal connective tissue cells undergo

an attempt at repair and the resulting proliferation of intimal connective tissues and ground substance is what leads to the fibrous thickening. This theory, as originally proposed, disregards any effect of insudated blood products or mural thrombi on the formation of the fibrous reaction. In discordance with this theory has been the rather conclusive evidence that the foam cells are not derived from fibroblasts but are altered smooth muscle cells and macrophages. However, there have been multiple variations of this hypothesis which incorporate some aspects of the thrombotic theory and more recent investigative findings, such as the lipid infiltration hypothesis discussed below.

Lipid Infiltration Hypothesis

Investigators near the turn of the century noted that almost any animal if fed high quantities of cholesterol and lipid in their diet will develop atherosclerotic lesions. As well, epidemiologic evidence suggested that the higher the cholesterol level in a population, the greater the incidence of atherosclerosis. Based on this, Anitschkow, in the early 1910's, advocated that the inciting cause of atherosclerosis is a high level of plasma cholesterol and that the disease is attributable to a disorder of lipid metabolism[75]. This theory was more one of etiology than pathogenesis and simply maintained that a high serum cholesterol has a toxic effect on the endothelium. The resultant change in the permeability characteristics of the endothelium allows for the deposition of lipids in the intima. The argument stopped at this point and the development of atherosclerotic lesions was then usually explained by some variation of Virchow's inflammatory theory.

There is no doubt that lipids are an important factor in the genesis of atherosclerosis[76-88]. Symptomatic atherosclerosis will very seldom develop in the presence of a low serum cholesterol. However, as the serum level of cholesterol rises, the probability of a myocardial infarction increases directly[79]. There also exists a group of inherited disorders of lipid metabolism which are associated with premature atherosclerosis, such as familial hypercholesterolemia[89]. Cholesterol supplemented diets are the rule rather than the exception in atherosclerosis research utilizing current animal models. Because of the importance of hyperlipidemia as a risk factor and as an experimental condition, it is relevant to digress for a brief description of lipid metabolism.

All lipids in the plasma circulate in combination with protein in particles referred to as lipoproteins. A lipoprotein consists of an outer hydrophilic layer of protein and phospholipid and an inner hydrophobic core of triglyceride and cholesterol. The outer layer imparts solubility to the otherwise insoluble lipid and cholesterol. There are several different categories of lipoproteins which can be separated by either ultracentrifugation or electrophoresis. Electrophoresis will separate the chylomicrons which remain at the origin from pre-beta-, beta-, and alpha-migrating peaks. An ultracentrifuge will separate the lipoproteins (which are lighter than plasma) into several groups - the chylomicrons (the lightest), the very low density lipoproteins (VLDL), the low density lipoproteins (LDL) and the high density lipoproteins (HDL). The separation techniques are complimentary as the VLDL corresponds to the pre-beta, the LDL with the beta and the HDL with the alpha fractions.

Each type of lipoprotein contains different amounts of cholesterol and triglyceride. Chylomicrons contain more than 95% triglyceride by weight, 1% cholesterol and less than 1% protein. VLDL contains more protein (10%), but still carries about five times as much triglyceride as cholesterol. LDL on the other hand is 50% to 60% cholesterol by weight and in man carries about 70% of the total plasma cholesterol. HDL is 50% protein by weight. Not only does the amount of protein vary between the different classes of lipoproteins but also the type of protein (referred to as apoproteins) varies.[90] There are at least eight different apoproteins currently identified. Apoprotein C is found in chylomicrons, VLDL, and HDL, whereas apoproteins A and D are found only in HDL. The B apoproteins are found in chylomicrons, VLDL and LDL which is why in abetalipoproteinemia (a rare inborn error of metabolism with lack of production of B apoproteins) no chylomicrons, VLDL or LDL are present in the plasma.

Each of the lipoproteins has a specific function. The chylomicrons are primarily involved in the transport of dietary triglyceride and cholesterol from the gut, through the lymphatics and thoracic duct into the blood stream and finally to the periphery. In the capillary beds of muscle and adipose tissue, lipoprotein lipase cleaves off glycerol and fatty acids from the chylomicrons for use in the cells. The remnants which remain are cleared from the bloodstream by the liver. This is the so called exogenous lipid transport system that handles dietary lipids and cholesterol.

There is a second pathway which is used to handle the endogenous transport of lipids and cholesterol. The liver synthesizes triglycerides and cholesterol and releases them into the bloodstream as

VLDL which is transported to the periphery[91]. A sequence of events then occurs whereby triglycerides and peptides are removed and the cholesterol ester content of the particle is increased, resulting in the formation of LDL lipoproteins. The LDL is an important vehicle for transporting the cholesterol to cells for use in membrane synthesis. Both the liver and extrahepatic tissues, including smooth muscle cells and macrophages, have receptors which can bind the LDL[92]. The cells in the body can thereby control the amount of cholesterol they take up by varying the number of LDL receptors on their surface[93,94]. Of major interest here is a familial disorder referred to as familial hypercholesterolemia (Type II hyperlipoproteinemia), where there is an absence of LDL receptors and rampant atherosclerosis. As will be outlined later there is an excellent animal model for this disease, the Watanabe Hereditable Hyperlipidemic (WHHL) rabbit.

The role of HDL has still not been reliably defined[95]. HDL has been shown to take up cholesterol from peripheral erythrocytes and there is some speculation that its role is to carry cholesterol from the periphery back to the liver[96]. The HDL levels have shown very interesting relationships with the most significant being an association with a decreased incidence of atherosclerosis. The HDL levels are higher in women, increased by estrogens, increased by moderate amounts of alcohol and exercise and decreased by obesity, diabetes, cigarette smoking and high carbohydrate diets.

As mentioned earlier, there is a direct correlation between the incidence of atherosclerosis and plasma LDL and an inverse relationship between the HDL levels and atherosclerosis[5,97,98]. It has been suggested that there is some abnormality in the metabolism of LDL which

causes the accumulation of lipid in atherosclerosis. These postulated abnormalities include defects in lysosomal function, lysosomal enzyme deficiency or unsuppressed LDL receptor synthesis. It has also been suggested that the mechanism whereby high levels of LDL cause atherosclerosis involves bypass of the LDL receptor in favor of bulk transport at high concentrations.

Insudation Theory

This theory was initially proposed by Rössle and restated by Doerr[99-104]. The basic premise is that injurious elements in the blood damage the endothelium or subendothelial connective tissues. This produces a local serous inflammation of the intima which is manifested as an insudate from the blood. Although the insudate is usually resorbed, avascular connective tissue may organize the insudate to form atherosclerotic lesions. This theory incorporates aspects of all the previous theories in its latter derivations, attributing the determining factor as to whether or not the insudate organizes in the presence and type of lipids in the plasma insudate.

Monoclonal Hypothesis

The monoclonal hypothesis proposes that the atheromatous plaques are neoplastic-like growths of wall myocytes in response to unknown tumorigenic influences[105,106]. This premise is based on observations originally made by Benditt who studied the distribution of A and B isoenzymes of X-linked glucose 6-phosphate dehydrogenase(G-6PD) in atheromatous plaques[107,108]. In uninvolved aortic intima, both isoenzymes were present, while in fibrous plaques, only one of either the A or B isoenzyme was predominately present. It was suggested that the smooth muscle cells in the plaque are similar to a leiomyoma and

result from the mutagenic effect of such things as exogenous chemicals, cholesterol or possibly a virus. Pearson studied the isoenzymes of fatty streaks and found that at a young age the lesions were ditypic though there appeared to be an intermediate lesion in which one type markedly dominated, suggesting that with time there is a selective survival of one or the other isoenzyme cell types with eventual monotypism[109]. The full significance of this observation is still not known however.

Discarded Theories

The local lipid synthesis theory was developed to explain the presence of lipid in the myocytes before it was known the myocytes were capable of phagocytosis[28,110]. It proposed that the lipid in plaques was synthesized locally by the myocytes themselves. There is no good evidence for this today.

The senescense theory holds that the smooth muscle proliferation in atherosclerosis is secondary to loss of normal growth control, possibly related to the age of the cell[111-114]. There is certainly good evidence to indicate that the fibrous plaque cells may possibly have already undergone numerous cell doublings by the time the plaque is formed ~~reflected~~ by their slower growth in cell culture[34,115]. There has also been some interest in growth inhibitors (which have been isolated from aortic walls) which may be locally deficient[116].

There have been multiple other theories ranging from anoxia of the vessel walls to neovascularization[117-119]. Many have been absorbed into other more inclusive theories whereas others have proven invalid.

Response to Injury Hypothesis

The last hypothesis to be discussed is certainly the most

comprehensive and explains the greatest number of observations. It was originally proposed by Ross and has since been widely presented in the literature[27,120-126]. An excellent review of the theory is presented by Ross in a recent issue of the New England Journal of Medicine.[27]

The response to injury hypothesis proposes that the major etiologic factor in atherosclerosis is some form of endothelial injury, either denuding or nondenuding[127]. Chronically elevated LDL levels are postulated to cause a nondenuding form of injury by changing the amount of cholesterol in the cell membranes with a resultant alteration of membrane viscosity or alternatively as a direct effect on the endothelium of LDL which has been oxidized by macrophages[128-130]. Some feel the endothelial damage may be immunologically mediated[131-133]. The injured endothelium then secretes growth factors which cause monocytes to adhere to the endothelium. With time the monocyte-macrophage migrates through the endothelium to a subendothelialial position to form a fatty streak. There is continued secretion of growth factors, the so called monocyte derived growth factors (MDGF), by the monocyte in this location. The underlying smooth muscle cells plus additional macrophages and epithelial cells respond to the chemotactic factors by migrating to the subendothelial area. This collection of cells and their products stimulates and injures the overlying endothelium leading to an eventual loss of cover. Consequent adherence of platelets and macrophages with release of their growth factors results in the eventual formation of fibrous plaques and, at a latter stage, the complicated lesions.

There are several aspects of this hypothesis which deserve closer scrutiny. The first is the role of the endothelium. It has long been

recognized that the endothelium is metabolically very active. The endothelium has been shown to produce vasoactive agents, growth factors and growth inhibitors so that it is not unlikely that it could have a role in the adherence of circulating monocytes[134-140]. The crux of the response to injury hypothesis is the eventual subendothelialization of the monocyte to produce a tissue macrophage. It may be that the macrophage does occasionally penetrate into the intima to act as a scavenger for various foreign materials but the evidence that this is the foundation of an atheromatous plaque is very weak. Much of the evidence to support this premise is based on one primate model which will be described in a latter section. Suffice it to say that this critical experimental work was done on monkeys fed a cholesterol enhanced diet and has not yet been verified in other laboratories. In a disease known to be multifactorial, this observation will require confirmation in the circumstances of an elevated endogenous rather than exogenous cholesterol environment and also in the presence of lower serum cholesterol levels than were used in the original experiment.

Once the monocyte turned macrophage is present in the subendothelial layer there is abundant evidence that it is capable of secreting numerous mitogens and chemotaxins[141-149]. Macrophages are capable of producing significant tissue destruction as there are several toxic substances which are present in their lysozomes including hydrolases and superoxide anions. The macrophage is thus more than capable of causing the eventual production of the observed lesion and destruction of the overlying endothelium.

Platelets have long been known to produce multiple different chemical compounds including several mitogens most notably platelet

derived growth factor (PDGF)[150-157]. The platelet, like the macrophage produces both mitogenic and chemotactic factors so that it also has the metabolic machinery to cause both the migration and proliferation of smooth muscle cells. Platelets adhere only transiently, if at all, to normal endothelium. They will respond to damaged endothelium or subendothelial connective tissue by adhering and releasing their granule contents[158]. The important role of platelets in the production of atherosclerotic lesions has been documented in various situations such as in the disease homocystinuria, after injury by intraarterial catheters and after bypass surgery at perianastomotic sites[159-164].

Acoording to the response to injury hypothesis the smooth muscle cells are considered to be quite passive in the production of the atheromatous lesions[165]. Although the smooth muscle cells are known to be present in the fibrous cap, they are felt to develop in this location as a response to the altered chemical environment produced by the products released from the macrophages and the platelets. They do contain receptors for LDL and will accumulate lipid to take on the appearance of foam cells. There is some evidence that the smooth muscle cells may exist in two different states, the usual contractile state and a synthetic state which may be capable of secreting a PDGF type of substance[166-168]. However, this work is preliminary and has not yet been substantiated.

Ross latter expanded his response to injury hypothesis to include a second pathway by which atheromatous plaques could develop. This was included to explain the relationship of hypertension, smoking and diabetes to atherosclerosis, as the original pathway is intimately

involved with hyperlipidemia. Rather than the growth factors being responsible for the migration of the various tissue elements produced by subendothelialized macrophages, it was felt that a sufficiently stimulated endothelium would have the ability to release sufficient growth factors on its own. These factors could then induce smooth muscle cell migration and proliferation with production of atheromatous lesions[120].

Summary

The attempts to unify all the varied observations on atherosclerosis into one all embracing theory of pathogenesis have been reviewed. There are multiple other forks of knowledge which are relevant to understanding atherosclerosis but have not been covered here. Active investigation of the part played by heparin[169,170], collagens[171], proteins in the cell wall[172,173], magnesium and potasium[174] in the production of atherosclerosis are being actively persued. Extrinsic factors acting on the arteries, such as the role of flow dynamics[175-177], as well as intrinsic factors such as vasospasm[178] and the role of prostaglandins are also generating interest. Some of these current areas of investigation will be touched upon in the following section with the examination of the animal models which are currently used for research in these areas.

Current Non-Rodent Models of Atherosclerosis

Up until this point, a concerted effort has been made to try and exclude results from animal experimentation of atherosclerosis in the discussion. This is an artificial and totally impossible situation as any research is necessarily based on animal models of the disease. The theories of pathogenesis are intimately tied to studies of the disease in an animal model. The reason so little is settled on the pathogenesis of atherosclerosis may be the inadequacy of current animal models to completely fulfil all criteria required for the perfect animal model. Some animals are good models for certain aspects of the disease but none present an identical representation of the human disease.

An ideal animal model should have several characteristics. The disease in the animal should have the same natural history as atherosclerosis in man, developing slowly over the course of the animal's lifetime with the same complications (for example, the occasional myocardial infarction or aneurysm formation). We would expect to see fatty streaks and atheromatous plaques affecting the same vessels as in man and having similar histological pictures and distributions. The incidence of the animal disease should share human epidemiological factors with an expected increase in males and the elderly. The lesions should develop without drastic environmental or dietary manipulations of the animal. Regarding the animal itself, it should be easy to acquire and inexpensive to maintain. To facilitate

manipulations, it should be a reasonable size and easy to handle. An animal with well defined genetic characteristics is a bonus as controls may be more valid.

Since the early 1900's, scientists have used a variety of animal species for atherosclerosis research. The earliest animal model research was done on the rabbit at the turn of the century. The rabbit continues to be popular but research is also done using other mammalian species, especially non-human primates, swine and rodents, plus avian and fish species. For the purpose of this presentation, I would like to concentrate on the rodents and this will be included as a separate section. Firstly, a brief description of current non-rodent animal models will be presented to tie in with the theories previously presented and review current areas of research. No attempt will be made to critically review all the animal work but rather a brief synopsis of current plus historical experimentation for each of the animal models will be presented. For further information there are several current detailed reviews in the literature[179-183].

Fish Models

Several species of fish including the Atlantic salmon[184-186], Pacific salmon[187-190], rainbow trout[190-194], steelhead trout[189,193-196], brook trout[197] and freshwater salmon[198] have been noted to develop degenerative lesions of their coronary arteries. In one study, up to 60% of steelhead trout caught prior to spawning had coronary arteriosclerosis and this increased to 90% of those caught at the time of spawning[195]. It has been found that the lesion incidence can be increased with dietary cholesterol supplementation. Fish have a naturally high level of cholesterol, about five times the human level,

and their lipoproteins are markedly different than human lipoproteins[199,200]. Because many of the lesions appear related to spawning with a higher incidence in males, most of the fish research has concentrated on the effects of steroid hormones on atherosclerosis.

There has not been a great deal of work on fish for several reasons. The disease progression is not similar to that seen in man and the coronary lesions are predominately myointimal hyperplasia. The fish have a grossly different anatomy from mammals and different metabolic pathways. The fish are not easy to handle and manipulate though they are quite cheap.

Avian Models

There has been a fair amount of study of birds, most especially pigeons[201-205]. Only certain strains of pigeons develop atherosclerotic lesions but the histology of the lesions is very similar to human atherosclerotic plaques both grossly and microscopically. Plaques are seen throughout the aorta, but most prominently in the thoracic aorta at the celiac bifurcation, somewhat more proximal than lesions seen in man. Coronary lesions do develop with occasional myocardial infarctions reported in pigeons. However, the etiology of the infarcts which occur is usually plaque embolization rather than intraplaque hemorrhage and thrombosis as is the case in humans[204].

The most studied breed of pigeons has been the White Carneau pigeon which develops lesions naturally on a normal diet[206-214]. Up to 30% of these pigeons have small foam cell lesions in the aorta as soon as one week after hatching and by twelve weeks of age 72% of the birds will have these lesions[201]. Grossly visible lesions are present in

about 30% of one year old White Carneau pigeons and nearly 100% of these birds by four years of age. Cholesterol will increase the rate of development, extent, and severity of the atherosclerotic lesions in all strains. Another White Carneau strain has been developed which differs from the random-bred strain in having a higher incidence of lesions[215]. Show Racer strains also exist which, although less susceptible to atherosclerosis than the White Carneau pigeons, have been selectively bred to develop two strains with either a hypo- and hyperresponsiveness to dietary cholesterol[216]. This genetic difference in susceptibility between strains has been correlated with biochemical differences in the aortic walls of the different strains[217-220].

The pigeons themselves are inexpensive, easy to handle, and a suitable size for manipulation. The availability of different relatively unaffected strains is an advantage.

The disadvantages of pigeons include the fact that they, like fish, are nonmammals a with different anatomy and metabolism than humans. Pigeons normally have high serum cholesterol concentrations with most of the cholesterol transported in high density lipoproteins rather than low density lipoproteins. Though the pigeons do develop lesions naturally, the fact that this occurs over a matter of years has led most investigators to use cholesterol enhanced diets to augment the incidence of lesions. Of interest, and as will be discussed more fully later, the lesions in the birds fed a cholesterol enhanced diet, though similar morphologically to the naturally occurring lesions, have a different histological appearance with more lipid and less fibrous tissue[221]. The coronary lesions which develop are in intramyocardial

branches rather than proximal vessels and the aortic lesions tend to be more proximal than is seen in man.

Other avian species have been examined although not to the extent that pigeons have been investigated. The lesions of the Japanese Quail even more closely resemble those of man histologically[222-225]. There are resistant and susceptible strains of the Japanese Quail. All strains show a higher lesion incidence in males. However, the lesions will develop only in the presence of a cholesterol enhanced diet. The same disadvantages as outlined for the pigeon exist for the Japanese Quail plus the fact its size is somewhat of a problem.

Chickens and turkeys have also been used for atherosclerosis research[226-231]. Lesions do develop naturally but with a low incidence so that dietary manipulation is the rule in most experiments which have been done. Though chickens do not usually die of the complications of atherosclerosis, the broad breasted bronze turkey may die of a dissecting aneurysm. One very interesting peculiarity is the increased incidence of atherosclerosis in animals which are infected with the herpes virus of Marek's disease[232,233]. This has been advocated as advantageous to investigate possible viral etiologies of atherosclerosis. However, it proves to be more of a deterent as the inability to control the incidence of the virus affects the results of experimentation in almost all circumstances and is a major problem in most labs. These animals are more expensive than pigeons and more difficult to handle with basically the same disadvantages.

Swine Models

Pigs are excellent animal models of atherosclerosis. They develop lesions which are histologically similar to both fatty streaks and

atherosclerotic plaques seen in man with much the same distribution and natural history[234-238]. The vessels are large which facilitates the observation of gross changes. There is a significant incidence of end organ lesions including aneurysms and myocardial infarctions. As well, the pig is one of the few animal models that develops intracranial lesions. Another important advantage, especially for investigation of the effect of lipids on atherosclerosis, is that the plasma lipoproteins of pigs are similar to those of man.

There has been a large amount of research done with the pig. Most of the studies have been descriptive in nature striving to examine the morphology of the lesions, sometimes in normal animals but most often in dietary manipulated animals[239-243]. It has been found that the uptake of Evans blue dye is increased in areas of the endothelium with increased permeability to proteins sparking interest in the concept of endothelial injury and its relationship to atherosclerosis[244-246]. Further studies to investigate the response to injury hypothesis have included characterization of chemotactic substances[247,248] and demonstration of a mononuclear cell infiltration of the intima[249]. Manipulations such as induced endothelial injury[250-252] and ingestion of antiprostaglandins[253] can also be investigated in the swine. Daoud has published several articles demonstrating lesion regression in the swine[254-257].

The major disadvantages of pigs are size and, in a fashion, its longevity. The pigs, like man, develop lesions over the course of their lifetime with an increased incidence in old age. Studies following naturally occurring lesions in pigs to twelve years of age have been done[258]. This does not affect descriptive experimentation

used to describe the lesions but it does affect manipulative experimentation, such as seeing the effect of different drugs on the incidence of lesions. The pigs are large and difficult to handle, and the cost of housing and maintaining a population which will give significant results is substantial. As well, by the time the experiment is completed, the question may have already been answered with a different method because of the length of time involved.

The size problem can be partially overcome by the use of miniature pigs[253,259-263]. Dietary manipulation is used to shorten the time period for the development of the lesions and to increase the incidence of lesions. This is very easy to do in the pig as the pig will eat a diet similar to common human diets. It has been found that a cholesterol and fat enriched diet will increase the incidence of lesions with a modest serum hypercholesterolemia and hyperlipoproteinemia. Unlike the case in pigeons, the dietary manipulations do not appear to change the histologic appearance and distribution of lesions[264]. However, there are marked changes in the apoprotein content of plasma lipoproteins with dietary manipulations[265].

There are certain strains of pigs which are affected by a von Willebrand like disease manifested by a bleeding disorder, a deficiency of von Willebrand's factor and reduced platelet retention[266-270]. These pigs have been found to be resistant to naturally occurring and dietary induced atherosclerosis. This has been postulated to be due to the platelet dysfunction and has been advocated as excellent evidence for the important role of platelets in atherogenesis.

Rabbit Models

The rabbit was the first animal used as a model for atherosclerosis. Ignatowsky reported his findings of atherosclerotic lesions in rabbits fed a diet of milk, meat and eggs in 1908[271]. Animal research did not get off on a good foot as Ignatowsky incorrectly deduced that the arterial lesions produced were the result of dietary fat rather than the cholesterol. Since that time there has been extensive investigation of the rabbit as the rabbit is inexpensive, easy to handle and maintain and reproduces rapidly.

The rabbit, in spite of its extensive use, is not a good model for atherosclerosis. The lesions have a different histology, beginning with medial smooth muscle necrosis and degeneration followed by cellular proliferation and medial calcification[272,273]. There may be intimal thickening or metaplasia in later stages but lipid is not normally found in the naturally occurring lesions. As well, the animals are herbivores with a different whole body cholesterol metabolism.

Natural lesions do not occur with a high incidence but dietary lesions can be induced easily with a high cholesterol and fat diet[274]. The usual supplementation includes a 1 to 3% cholesterol and 4 to 8% fat diet which gives extremely high serum cholesterol levels in the range of 1000 - 3000 mgm%. If only a cholesterol supplemented diet without fat supplementaion is fed to the rabbits, the lesions which develop are more severe, presumably because of mobilization of the more saturated endogenous fat stores[275]. Although the fat and cholesterol diet regime will result in the deposition of foam cells, fibrous tissue and smooth muscle cells in the

intima, fibromuscular caps are unusual. The distribution of the lesions differs from humans in that the aortic lesions are more proximal and the coronary lesions spare the large proximal arteries in favor of the smaller intramyocardial branches. Lesion complications are unusual. The effect on the animal includes not only the vessel changes but also a lipid storage type of disease with fat and cholesterol accumulation in many other organs, such as the liver, spleen, bone marrow, adrenal glands and eyes[276].

In spite of the noncomparability of lesions there continues to be a great deal of interest in rabbits. A substantial portion of the studies concentrate on endothelial characteristics and the effect of artificial damage to the endothelium of cholesterol fed rabbits[277-287]. Ingestion of antiplatelet medication[288-290], calcium channel blockers[291-297], and propranolol[298] have all been used to try and alter lesion formation. Prostoglandins are currently in vogue as a topic of study and are being investigated in the rabbit[299-301]. Rabbits have been subjected to vasectomy[302], partial ileal bypass[303], and radiation[304] to examine the effects on lesions. An interesting observation concerns the immunologic vascular injuries which can be induced by repeated injection of foreign serum proteins[305,306]. These lesions more closely resemble human lesions and occur in more analogous locations to man. This has lead to speculation that this model may be useful to study immune complex endothelial damage.

An extremely important study was recently done by Wilson and colleagues[307]. Rather than the cholesterol and fat augmentation of the diets usually used in the past, the amount of fat and cholesterol

in the diets of rabbits in this study were similar to the fat and cholesterol content of human diets. The serum cholesterol levels were found to be lower, ranging from 138 to 532 mgm/dl. After five years on this diet proliferative atherosclerotic lesions developed in which fibromuscular plaques were found to be present. There are several other studies which document changes in the nature of lesions found in rabbits fed lower cholesterol diets[308,309]. This may not improve the use of rabbits as a model for atherosclerosis but it does serve to raise some very serious questions about the assumption that the lesions which develop in the presence of high dietary cholesterol resemble naturally occurring lesions in regards to morphology and pathogenesis.

Prior to concluding the discussion of rabbits and atherosclerosis, a special case should be examined. Watanabe developed a strain of rabbits by selective inbreeding which had markedly elevated levels of LDL and developed rampant atherosclerosis[310]. The strain became known as the Watanabe Hereditable Hyperlipidemic (WHHL) rabbit and it was investigation of this strain that lead to a Nobel Prize for Goldstein and Brown[89,93,94,311,312]. The strain was used to deduce the presence of LDL receptors and clarify many aspects of lipoprotein metabolism. These rabbits, like individuals afflicted with familial hypercholesterolemia (Type II hyperlipidemia), have a genetic deficiency of LDL receptors which leads to an elevated LDL, and consequentially cholesterol, in the serum. Electron microscopy of the atherosclerotic lesions present in the aortas of WHHL rabbits has demonstrated smooth muscle cells and lipid laden foam cells in the intima. The cholesterol levels in the WHHL rabbit are extremely high and whether or not the results from this model can be generalized to

the human atherosclerotic condition which develops in the presence of lower serum cholesterol levels remains to be seen. Certainly the WHHL rabbit provides an indispensable model for familial hyperlipidemia.

Non-human Primate Models

The primate model presents nearly the complete reverse scenario as the rabbit model. For the same reasons that rabbits are considered good models, the primates are considered bad models. The animals are very expensive to maintain and acquire. Several of the species are considered endangered and supply difficulties are a major problem. The animals are often very difficult to handle. Alternatively, for the same reasons that rabbits are poor models, the primates are excellent models. Being phylogenetically close to man, they have similar lipoproteins and cholesterol metabolism[313,314]. The lesions are histologically similar to those of man and are present in much the same distribution. Complications such as aneurysms and myocardial infarctions are documented to occur[315-317]. The incidence of lesions can be increased with cholesterol supplemented diets. Interestingly enough, in the 1930's it was felt that monkeys were poor models and resistant to dietary cholesterol induced lesions[318]. Though this concern has since been shown to be unwarrented, it did significantly delay atherosclerosis research in the primate model.

The new world monkeys have been the most extensively studied[319-323]. There have been studies of stumptail macaques (Macaca arctoides)[324], Rhesus monkeys (Macaca mulatta)[325-332], cynomolgus macaques (Macaca fasisularis)[333-339], pigtail macaques (Macaca nemestrina)[340,341], squirrel monkeys (Saimiri sciurens)[342-346], baboons[347-353] and African green monkeys

(Cercopithecus aethiops)[354-357]. The number of studies and number of animals in each study has been limited because of the economic and supply concerns previously alluded to. In order to get results as quickly as possible, whether for economic or time concerns, the animals in most of these investigations were put on cholesterol supplemented diets to increase the incidence of the lesions. The assumption is always made that this does not change the nature of the lesion itself.

A number of studies on monkeys have come from Ross's group in Seattle, Washington. Part of the reason is that this institution houses the largest breeding colony of pigtail monkeys in the world. The colony has been in existence for twenty years and contains some three thousand animals.

Crucial to Ross's response to injury hypothesis is the subendothelial migration of monocytes from the blood to become tissue macrophages. This work is based on observations made on pigtail monkeys[358,359]. Animals for the study in which these observations were made were selected on the basis of their response to a cholesterol and fat supplemented diet. Only animals that developed a total cholesterol level of between 300 mg/dl and 500 mgm/dl after being placed on a trial high fat and cholesterol diet were selected for the study. Fourteen animals were thus selected and placed back on the fat and cholesterol supplemented diet. The animals were then sequentially sacrificed at varying intervals and their aortas examined grossly, by light microscopy, by scanning electron microscopy and also by transmission electron microscopy. It was under these circumstances that macrophages were observed penetrating through the endothelium. The papers in which these findings were presented should be reviewed to

examine the excellent micrographs obtained and review the concise narrative of the sequential changes observed with time following the cholesterol diet implementation in these monkeys. A major concern with this study is the use of a cholesterol supplemented diet. In a separate study, Bond [341] compared the histology of coronary lesions in cholesterol fed pigtail macaques (the same primate used by Ross) and control monkeys which were fed a normal diet. He found that in control animals the lipid in coronary plaques was entirely intracellular whereas in the animals fed the atherogenic diet lipid there was substantial extracellular lipid. Therefore though Ross's experiment is very well done the findings cannot necessarily be generalized to occur in nonhypercholesterolemic states or circumstances where the elevated cholesterol is presented through the endogenous pathway rather than the exogenous pathway. It is entirely possible that the morphology and pathogenesis of lesions produced under these two circumstances may be quite different.

There is evidence in not only the monkey but also in the rabbit and pigeon that the lesions in cholesterol fed animals are often quite different than those found in noncholesterol fed animals. It is also relevant to recall that there are two different methods of handling cholesterol in the body - the endogenous and exogenous pathway. It has been suggested that exogenous cholesterol causes atherosclerosis by blocking LDL receptors and thus affecting the endogenous pathway[274]. However, it may be possible that cholesterol in the two different pathways affects the atherosclerotic process in different ways. This has not yet been established or ruled out. Until it is, though the evidence put forward by Ross remains an excellent description of the

disease in the presence of hypercholesterolemia, the concept of subendothelialization remains a contentious issue in noncholesterol fed animals. Certainly the findings may have relevance to the lesions in that subset of human disease where individuals have a significantly elevated serum cholesterol secondary to dietary ingestion, a well recognized risk factor. However, it must be remembered that the vast majority of afflicted individuals have serum cholesterol and lipid profiles that are not significantly different from unaffected persons. Though important, hypercholesterolemia is not the only risk factor, with cigarette smoking and hypertension being equally as important. The reason cigarette smoking and hypertension are associated with an increased incidence of atherosclerosis has not yet been adequately explained.

Other Mammal Models

There have been several other animals used on occassion to study atherosclerosis, none of which have proven to be of any real lasting value as an animal model. These range from cows[360,361] to Aleutian disease of the mink[362] to Ricketsial disease in the guinea pig[363,364]. Though dogs are a very popular laboratory animal, they have not proven suitable for atherosclerosis research[365-372]. It has been found that severe experimental conditions including not only dietary manipulations but often also thyroidectomies are required to produce a significant number of atheromatous lesions in dogs. The lesions that do form are quite different in that they are mainly sclerotic without any lipid, they have a different distribution than that seen in humans and, as a rule, no complications of the lesions develop.

Rodent Models of Atherosclerosis

Rodents are extremely popular laboratory animals. They are inexpensive to acquire and maintain. They are widely available and reproduce rapidly. Because they are so popular, there is an abundant literature available on the characteristics of multiple different strains whuch have been developed each of which often presents useful genetic differences.

A search for a suitable rodent model of atherosclerosis has a long history. In 1938 Wilens described spontaneous cardiovascular disease in rats though the lesions he observed did not very closely resemble human atherosclerotic plaques[373]. Experimentation up to 1956 was reviewed by Filios who noted that attempts to produce atherosclerosis experimentally in rats with cholesterol feeding alone produced either no lesions, lesions consisting solely of lipid infiltration of various layers of the arterial wall, or lipid containing lesions with some intimal proliferation[374]. He maintained that a rat diet supplemented with sodium cholate and thiouracil, in addition to the cholesterol, would produce intimal plaques with lipid present both extracellularly and within foam cells which more closely resembled the human condition. There was general agreement at the time that the rat was not a good model of atherosclerosis because the infrequent naturally occurring lesions which were observed did not resemble atheromatous plaques[375]. It is interesting that this same concern existed for the better studied rabbit model. The small body and artery size of the rat also hindered experimentation.

Because of the ideal nature of rodents as laboratory animals, there

continued to be sporadic investigation of the rat as a model for atherosclerosis. As obestity is often associated with atherosclerosis, the search for a model for atherosclerosis was often accompanied by examination of obese phenotypes. It was noted that obesity in the rat could be produced by hypothalamic lesions[376], and genetically obese rats were developed by selective breeding[377]. However, none of these rat models developed a significant incidence of atherosclerotic lesions.

The effects of different dietary manipulations on the rats was also investigated[378,379]. Gresham[379] showed that piebald rats fed not only cholesterol and cholic acid, but also a 40% butter enriched diet had a high incidence of myocardial and renal infarcts but no atherosclerosis. However, if the diets were supplemented with 40% arachis oil instead of butter, no infarcts were noted but extensive atherosclerosis developed. Even the effect of emotional stress and its relationship with myocardial necrosis was studied in rats[380].

It became apparent that atherosclerotic lesions varied between species, not only in incidence and distribution but also in histology. The irradiated CFI mouse developed lesions characterized by medial degeneration and calcification[381] whereas the cholesterol fed Wistar rat developed lesions which were more similar to human atherosclerotic plaques[382]. Concern then shifted towards examination of different natural species and selectively bred strains which would develop human like atherosclerotic lesions with a high incidence. This investigation was intensified when it became apparent that the use of scanning and transmission electron microscopy would overcome some of the disadvantages of using the small rodent aorta[383-385].

Wild rodents were placed on high fat and cholesterol diets to examine the vascular response. Dietrich[386] examined five different species (three mouse strains, the meadow vole and the collared lemming) and found that half the animals died on the cholesterol and fat supplemented diet he had choosen to use, with no significant production of aortic lesions. Wistar rats[382,387,388] and Sprague Dawley rats[389-392] also attracted a fair amount of interest. Peric Golia and Peric Golia[391] demonstrated the rare intimal plaque which could be demontrated in virgin male Sprague Dawley rats if allowed to reach an advanced age. However, though lesions can be produced in these common laboratory rats with artificial dietary conditions, interest remains diminished because of the low incidence and different histology of the lesions.

Vascular lesions have been demonstrated to occur in rodents with potentially interesting genetic disorders. Tiel[393] fed a cholesterol enhanced diet to fawn-hooded rats with platelet storage pool deficiency and was able to produce aortic lesions. This model may have potential for investigation of the role of platelet mitogenic factors in atherosclerosis since platelets from these rodents lack these factors.

Various manipulations of the rodents have been reported in the literature in an attempt to develop better models. The demonstration of atherosclerotic lesions in rats made diabetic with either streptozotocin[394] or alloxane[395] are reported. Deoxycorticosterone has also been used to induce cardiac and renal lesions[396].

Breeding experiments were conducted to select strains of rodents which are more susceptible to atherosclerosis. Various obese rodent strains were developed as a result of different breeding

experiments[397-400] as well as strains which are more susceptible to an atherogenic diet[401-403]. In 1963, Okomoto reported a strain of spontaneously hypertensive rats (SHR) developed by inbreeding of Wistar rats[404,405]. Though some intimal changes were observed in old rats of this strain[406,407] the strain itself did not prove to be a good atherosclerosis model. However, in 1973 Koletsky crossed these SHR rats with Sprague Dawley rats and observed a very interesting phenotype[408]. Certain rats demonstrated marked obesity, hypertension, hyperlipidemia, endocrine and metabolic disturbances and premature atherosclerosis on a normal diet. Prior to a more complete discussion of the Koletsky rats, I would first like to examine another obese inbred rodent strain developed at about the same time, the Zucker-fatty rat.

Zucker Rats

In 1961, Zucker reported a new strain of obese rats which were developed by inbreeding of a spontaneous mutant in 13M rat stock[409-412]. The fatty body morphology in this rat is transmitted as a single recessive gene (fa) so that the rodents will express the fatty phenotype in the homozygous recessive state (fa/fa). The heterozygous (Fa/fa) and homozygous dominant (Fa/Fa) genotypes do not express the fatty trait. Although there were certain strains of obese mice reported to this time, this was the first rat reported which develops obesity without requiring creation of a hypothalamic lesion.

The level of fatty acids and cholesterol in the serum of the fatty rats is significantly elevated above the level in non-Zucker rat controls. However, in spite of these lipid abnormalities, aortas at one year of age do not show any evidence of atheromatous plaques.

Several investigators have confirmed this absence of atherosclerosis in the fatty rat. The inclusion of this particular rat model in this discussion stems from the fact that though it has similar metabolic changes to the Koletsky rat, it does not, like the Koletsky rat, develop atherosclerotic lesions. The answer to why the Koletsky rat forms atherosclerotic lesions in the presence of elevated lipids and cholesterol but the Zucker rat does not, may be an important piece of the puzzle that is the pathogenesis of atherosclerosis[413].

The lipoprotein status of the Zucker rat has been extensively investigated[414-426]. The serum cholesterol is slightly elevated and triglycerides are substantially elevated. The hypertriglyceridemia is attributed to an elevated hepatic production of VLDL. The LDL and HDL levels are only slightly raised (about two times normal) in comparison to a major increase in VLDL. The level of lipoproteins increases with age and a full grown animal has a cholesterol level two to three times that of a heterozygote control and a triglyceride level five to six times the value of a control heterozygote animal.

There are widespread endocrine abnormalities. Though the fatty rats are hyperinsulinemic, with an elevated secretion of insulin from the pancreatic islets, their blood glucose is normal. The pancreatic islets themselves are increased in both size and number in the Zucker rat. Though the level of glucagon in the pancreas is normal, the circulating serum level is depressed. There is a decreased secretion of gonadotropins by the pituitary which is reflected by an impaired reproductive function. Fatty animals are marginally hypothyroid.

There is a generalized increase in the activity of the gluconeogenic enzymes. Lipogenesis from glucose is brisk in young

fatties though it does decrease with age. Though fatty rats are hyperphagic, the large increase in the number of adipocytes is also related to a greater increase in body fat per gram of food ingested which is seen in all Zuckers in comparison with other rats. The large increase in the number of adipocytes can be partially decreased but not prevented with dietary manipulation[427]. Likewise, exercise will only moderately decrease the accumulation of adipose tissue[428]. It may be that defective brown adipose tissue oxygen consumption is responsible for some of these changes in fat accumulation[429].

Koletsky Rats

The Koletsky strain, as mentioned earlier, was developed from selective inbreeding of mutations occurring in a cross between SHR rats and Sprague Dawley rats. The Koletsky rats resemble the Zucker rats as they also develop obesity, hyperlipidemia and endocrine disturbances with the expression of a homozygous recessive genotype[408,430-436]. The affected rats are markedly obese, increasing in weight from 200 grams at six weeks of age to 680 to 1000 grams at eight to nine months of age. The lipid profile is very similar to that demonstrated by the Zucker rat, with a marked elevation of triglycerides in the form of VLDL and a mild elevation of serum cholesterol. The endocrine changes and marked hyperplasia of the Islets of Langerhans which are present in the Zucker rat are also present in these rats.

Unlike the Zucker rats, the Koletsky rats which have the recessive trait are hypertensive and develop lesions of their arteries. Koletsky [408] was able to identify gross vascular disease in fifty percent of the animals he studied. The lesions he identified were most prominent in the muscular mesenteric, pancreatic and hepatic arteries. The

lesions result in gross, prominent enlargement of the arteries with both focal and diffuse nodular thickening, beading and tortuosity. Affected arteries were often dilated and thrombosed. The lesions stained for fat with Oil Red O stain. By light microscopy, the lesions were identified as intimal fibrous plaques formed by proliferating smooth muscle cells. Lipid was present in both intracellular and extracellular locations. The extracellular lipid accumulation was at times extensive, accumulating to form pools of fat associated with the fatty plaque formation in the intima and/or media. Three of the thirty two rats in this study died of a ruptured abdominal arteriosclerotic aneurysm. Koletsky later reported [430] the presence of a polyarteritis in unaffected arteries which was attributed to the hypertensive state. He also noted that the nonobese heterozygote rats also developed vascular disease though to a lesser degree and later in life.

Renal disease also develops in these obese rats. Both glomerulonephritis and nepherosclerosis develop in the kidneys which is manifested by a marked proteinuria and hyperuricemia. An interesting and unexpected finding is that the amount of proteinuria correlates directly with the degree of hyperlipidemia. Renal disease is the most frequent cause of death in the animals resulting in a short average life span of only ten months.

The mutant recessive gene responsible for the obese phenotype was referred to as the cp gene with the dominant gene referred to as the + gene. The heterozygotes (cp/+) are required to maintain the population as the affected homozygote (cp/cp) recessive rats are unable to breed, as much for mechanical reasons as endocrine reasons. Thus, the

affected homozygote recessive animals (cp/cp) are the result of crosses between unaffected heterozygotes (cp/+).

Because of difficulties in maintaining this strain due to the kidney disease and resultant early death, two related strains were developed by Hansen at the National Institutes of Health (NIH) in Bethesda, Maryland[437]. The first strain was developed by crossing the Koletsky rat with the LA/N rat[438]. The LA/N rat is an inbred normotensive strain at the NIH which was developed from a cross between an Albany rat (Alb/N) and a hooded strain of unknown origin. Multiple backcrosses were carried out to eliminate the noncorpulant genes of the Koletsky strain. The resultant rat strain, known as the LA/N - corpulent (cp) rats, retains the abnormal serum lipid profile and the propensity to form atherosclerotic lesions of the aorta. The University of Alberta LA/N-cp colony was developed from a gift of breeding stock from the fifth backcross of the twelve total backcrosses which were done at the NIH.

The second strain derived from the Koletsky rats was developed by crossing the Koletsky rat with a spontaneously hypertensive rat (SHR/N) at the NIH. The SHR/N rat had been developed from the original Okatomoto SHR strain, one of the two original parent strains of the Koletsky rat[439]. Multiple backcrosses were again undertaken to eliminate the noncorpulent genes of the Koletsky strain although in this instance the hypertensive genes were not bred out of the strain. This rat, known as the SHR/N - corpulent (cp) rat, is used as a model for non-insulin dependent diabetes of humans. Several investigators have suggested that the SHR/N-cp rat maintains the lipid and vessel abnormalities of the parent strain, though the information is sketchy

at best[440-443].

U of A Rat Colony

The University of Alberta colony of the LA/N-cp rat, as mentioned, was developed from rats of the fifth backcross between the LA/N rat and the Koletsky rat. The strain is now firmly established and data is starting to accumulate at a rapid pace. Several major papers have been published[444-446] or are pending publication[447-450]. Most of the effort to date has involved the identification and quantification of the arterial and myocardial lesions which occur in the LA/N-cp rat plus development of a lipid profile.

The homozygous recessive rats (cp/cp) are obese with an average adult body weight at nine monthes of age of 850 grams compared with a weight of 400 grams for the heterozygote (cp/+) at the same age. The obesity of the corpulent (cp/cp) rats is a reflection of their hyperphagous behavior though the weight difference between lean and obese rats cannot be totally eliminated by pair feeding the animals suggesting there are other factors involved in the development of the obesity[445]. The rats are not hypertensive and the systolic blood pressure in lean and obese animals averages 120 to 140 mm Hg by tail cuff.

Both homozygous (cp/cp) and heterozygous (cp/+) rats develop lesions on their aortas though the lesions are more frquent and severe in the corpulent animals. Scanning electron microscopy has been used to examine and quantitate the lesions of the arterial system[444]. All lesions which have been reported in other species[206,278,280,328] have been demonstrated to occur in the LA/N-cp rat. This includes dead endothelial cells, cells with raised or missing nuclei, polygonal cells

and areas of desquamation with attached blood elements. Frank ulcerated lesions and evidence of an old rechannelized thrombus have also been identified. Arterial damage has been shown to be common in the aortic arch, less common in the distal aorta and minimal in the renal arteries. Severe lesions were most commonly found in the superior mesenteric and pancreatic arteries. Occlusive coronary thrombi and areas of myocardial necrosis have also been identified in corpulent animals[446]. Myocardial infarctions have been documented to occur in these animals.

Like the Koletsky strain, the corpulent rats (cp/cp) have the equivalent of human Type IV hyperlipidemia with markedly elevated triglycerides and mildly elevated cholesterol. Plasma triglycerides in the affected homozygote (cp/cp) average 330 mgm% compared with 40 mgm% in the heterozygotes (cp/+) while the corresponding values for serum cholesterol are 215 mgm% and 80 mgm% respectively. The lipoprotein electrophoresis of serum from lean rats shows a normal rat lipid and lipoprotein status with most of the cholesterol and phospholipids carried in the HDL portion of the serum. The corpulent rats demonstrate a marked elevation of VLDL with very interesting sex differences[448]. In young corpulent females the VLDL is more elevated than corpulent males of the corresponding age. From this point on, the values diverge even further as the male values decrease to approach control values while the female VLDL levels continue to rise. This does not correlate at all with the nature of the lesions, as the corpulent male rats develop lesions at an early age at a time when the females are relatively unaffected and it is only latter in life that the females develop a significant incidence of arterial lesions.

What the lesion incidence does correlate with is the degree of insulin resistance[447]. Although the fasting glucose concentrations of corpulent rats are in the normal range the animals are hyperinsulinemic and have impaired glucose tolerance tests. Insulin concentrations are only mildly elevated in the relatively protected female but markedly elevated in the more disease prone male. Both sexes show hyperplasia and hypertrophy of the Islets of Langerhans. This would seem to indicated that hyperlipidemia may be a necessary condition for the development of atherosclerotic disease but is not in itself a sufficient condition.

It is important to remember that all these changes occur while on a normal rat chow diet without any fat or cholesterol supplementation. The hypercholesterolemia that develops is therefore due to changes in the endogenous cholesterol pathway. The WHHL rabbit is the only other model which consistently demonstrates this phenomena though the cholesterol levels in the WHHL rabbit are strikingly elevated compared to the more moderate elevation in the LA/N-cp rat.

Lingering Doubts and Research Hypothesis

The basic underlying problem continues to be that the current models for atherosclerosis have major problems associated with their use in research on atherosclerosis. Birds and fish are not mammels. Swine are large and difficult to handle. The histology of the lesions which occur in rabbits is dissimilar to that which occurs in man. Monkeys are expensive to acquire and maintain.

On the other hand, of course, each animal model has certain

advantages. The pigeons develop a high incidence of plaques which do have a histological relationship with human atheromatous plaques. The lesions in the swine are even more similar histologically to human plaques. Rabbits are excellent laboratory animals with good sized vessels for manipulation and observation. The monkeys, being phylogenetically closest to man, have similar lipoproteins in addition to a parallel type of arterial disease.

There are also separate models which demonstrate certain characteristics advantageous to experimentation on certain aspects of atherosclerosis. This includes the swine which develop von Willebrand disease[266-270], used to investigate the role of platelets in atherosclerosis, and the WHHL rabbit[310-312], an excellent model of familial hyperlipidemia. The immunologic injury of vessels produced in the rabbit model[305,306] is another example of the use of a given model for experimentation on a particular angle of this highly complex disease.

However, the major problem appears to be the necessity to supplement the laboratory diets with cholesterol in order for the animals to develop lesions at a significant rate. For some animals, such as the rabbit, lesions will not develop in the absence of dietary supplementation. In others, such as the primates, lesions do develop spontaneously but do so with a low frequency and predominately in animals at an advanced age.

The handling of cholesterol presented in the diet is quite separate from the handling of endogenous cholesterol. Dietary cholesterol is transported in the chylomicrons and VLDL whereas endogenous cholesterol is transported in the LDL fraction. The assumption that disease in the

presence of the dietary cholesterol is identical to endogenous disease may not be justified. Evidence for this has been presented in pigeons [221], swine [265], rabbits [307-309] and primates[341].

Rats are the most commonly used laboratory animal for several reasons. They are inexpensive to acquire and maintain and are easy to manage in the lab. Because they are so commonly used in laboratories, they have been well characterized in terms of their anatomy and metabolism. Because the genetic characteristics have been so well defined, the presence of genetically similar controls for most strains is certainly an advantage.

The LA/N-cp rat shares these advantages but also shares many of the disadvantages posed by use of a rat model. Rats have a different lipoprotein profile with the serum cholesterol carried predominately in HDL rather than LDL which is the case in humans. The rats have small vessels although this is less of a problem with more recent techniques utilizing electron microscopy.

The LA/N-cp rat presents several advantages which would suggest it has the potential to be an excellent model of atherosclerosis. The more moderate elevation in serum cholesterol, which resembles Type IV human hyperlipidemia with a predominate increase in VLDL, is a better representation of the more common state of human hypercholesterolemia than exists in other animals which tend to manifest a much higher serum cholesterol under experimental conditions. The arterial lesions observed by SEM are similar to atherosclerotic lesions observed in many other animals. The lesions occur with a high incidence in affected animals. As in man, the aorta and major abdominal vessels are involved with relative sparing of the renal arteries. The lesion incidence

shows a significant sex difference with male predominance similar to the case in humans, plus a fascinating relationship with the degree of hyperinsulinemia. Understanding of the exact nature of this relationship is still evolving. The biggest advantage with the LA/N-cp rat model is that the lesions develop in the absence of dietary or environmental manipulations. However, in spite of the work which has already been done, the lesions have not been examined histologically to verify their similarity to the lesions found in man.

Very briefly I would like to pose several questions which form the basis for my current research project:

1. Are the lesions in the University of Alberta LA/N-cp rats similar histologically to human atherosclerotic lesions?
2. Is there any evidence for macrophage subendothelialization in this animal model which has endogenous hypercholesterolemia rather than dietary hypercholesterolemia?
3. Are there age differences in the nature of the arterial lesions in this animal model? i.e. is there any evident progression of lesions?

My working hypothesis is that the LA/N-cp rat is a good model of human atherosclerosis. The animal is inexpensive to acquire and maintain, easy to handle and develops a significant incidence of arterial lesions. I propose to address the questions outlined above by using light microscopy, transmission electron microscopy and scanning electron microscopy to examine the arterial lesions of the LA/N-cp rat.

EXPERIMENTAL DESIGN

LA/N-cp Rat Colony

The LA/N-cp rats used in this study were bred in the University of Alberta colony. The original stock were from the fifth backcross between the LA/N rat and the Koletsky rat at the National Institutes of Health, Bethesda, Maryland. The expression of the corpulent trait is controlled by a homozygous recessive gene. As the homozygous recessive animals are incapable of breeding for functional more than hormonal reasons, the homozygous recessive corpulent animals are derived from matings between two heterozygous parents. At the time of weaning, the animals are easily classified as either corpulent (cp/cp) or lean (either cp/+ or +/+) by palpation of the subcutaneous abdominal fat pads which are enlarged in the cp/cp rats. The homozygous normal (+/+) condition is determined by trial breeding with a known heterozygote. The animals used in this experiment were both homozygous normal (+/+) and homozygous corpulent (cp/cp) male rats. Four rats from each group at both six and fourteen months of age were selected.

At the time of weaning at four weeks of age, all experimental rats were housed individually in 48 x 26 x 16 cm polycarbonate cages fitted with stainless steel wire tops. The lights were maintained on a reversed 12/12 hour light cycle with lights on at 04:00h and off at 16:00h. Room temperature was maintained at 20°C with a relative humidity of 40-50%. Other than for routine cage cleaning and maintenance, the rats were not manipulated in any other manner.

Tap water was continuously available. The rats were fed ad lib with rodent laboratory chow (Wayne Lab Blox, Allied Mills Inc., Chicago IL 60601). This pelleted diet which is composed of grains and other plant products contains approximately 4% total fat and virtually no cholesterol.

Specimen Preparation

The rats were anesthetized in their own cage with 4% halothane in room air and this was continued on the operating bench with a nose cone placed over the facial area through which 2-3% halothane was delivered. Prior to commencing any procedure, the depth of anesthesia was taken to the point where the animals would not have a withdrawal response to pinching the feet or any other painful stimulus.

The abdomen was opened in the midline to the xyphoid. The sternum was then divided in the midline and the attachments of the diaphragm to the anterior chest wall divided. The left ventricle was punctured with an 18 gauge needle which was connected to the perfusion apparatus.

The perfusion apparatus connected the 18 gauge needle via a sidearm T-piece to a three way stopcock. The T piece sidearm connected the line to a pressure transducer, the signal from which was fed into a Beckman recorder. After modulation and amplification of the signal, the output was directed to a voltmeter with the output displayed in millivolts. Prior to use of the perfusion apparatus, the system was calibrated by connecting the needle to a manometer. After the line was flushed of any air bubbles, a zero level was set. Fluid at a known pressure was then infused and the gain adjusted so that the output on

the voltmeter corresponded in millivolts to the pressure reading on the manometer.

The three way stopcock was connected to two separate volume containers, one of which contained the perfusate and the other of which contained the perfusate plus fixative. One could adjust the stopcock to select either perfusate, perfusate plus fixative or no flow. The volume containers were closed systems so that pressure could be applied to the top of the fluid columns. This pressure load was derived from the source supply which passed through a pressure regulator so that the amount of pressure applied could be adjusted. After passing through the regulator, the output was split with a Y connector so that the pressure on both fluid columns was equal.

The perfusate utilized in the experiment was Tyrode's solution. This physiologic solution contains 100 mgm/l $MgCl_2*6H_2O$, 200 mgm/l KCl, 1000 mgm/l $NaHCO_3$, 8000 mgm/l NaCl, 50 mgm/l $NaH_2PO_4*H_2O$, 1000 mgm/l glucose and 200 mgm/l $CaCl_2$. The perfusate plus fixative contained Tyrode's solution plus 2.5% electron microscopy grade glutaraldehyde which was made by adding 100 cc of 25% stock glutaraldehyde to 900 cc of Tyrode's solution.

Once the 18 gauge needle was placed into the left ventricle, perfusion was commenced with Tyrode's solution, maintaining a constant perfusion pressure of 100 mm Hg (100 mV on the voltmeter). The right atrium was incised to allow the egress of fluid from the vascular system. A total of 200 cc of Tyrode's solution was perfused by which time the fluid ran clear from the atrium. At this time the perfusate was changed to the Tyrode's solution plus 2.5% glutaraldehyde and a further 200 cc was infused, again with careful monitoring to maintain

the perfusion pressure at 100 mm Hg. By this time the rat's corpse was well fixed.

The aortic arch was then carefully dissected out and removed from the animal. The arch was divided into two mirror image portions by dividing the arch through the superior and inferior walls. This entailed division of the three major branches of the arch so that the inside of both the arch and these branches was visible. One half was immediately placed into a vial containing 2.5% glutaraldehyde in Milloning's buffer. The other half was divided into three approximately equal portions and likewise placed into the same solution. Milloning's buffer contains 16800 mgm/l NaH_2PO_4, 385 mgm/l NaOH, 5400 mgm/l glucose and 50 mgm/l $CaCl_2$ buffered to a pH of 7.2.

After a minimum of two days in the glutaraldehyde solution, all samples were washed three times with Milloning's buffer for fifteen minutes each time. The specimens were then placed in a 1% solution of osmium tetroxide for sixty minutes. The aortic samples were dehydrated in a graded ethanol series (50%, 70%, 80%, 90% and 100%). The larger intact half of the aorta was critical point dried (See Vac Inc., Pittsburgh, PA) and mounted on an aluminum stub with silver glue. The sample was sputter coated with gold (Model 5150B Sputter Coater, Edwards High Vacuum, Crawley, U.K.) and was ready for observation in the scanning electron microscope (SEM).

After the graded ethanol dehydration, the three smaller portions were immersed three times in 100% propylene oxide for ten minutes each time. From this they were transferred to 50% propylene oxide plus 50% epon (Araldite CY212) for three hours and then into fresh 100% epon

overnight. The next day each sample was placed in a separate block with epon and the samples placed in an oven at 60°C for twenty-four hours. At this point in addition to the sixteen SEM aortic arch specimens (one each from the four six month corpulents, the four fourteen month corpulents, the four six month leans and the four fourteen month leans) there were forty eight epon blocks containing segments of the aortic arch (three each from the same animals) ready for sectioning.

For each animal, two of the three epon blocks were randomly selected for sectioning. For each block, a razor blade was used to trim the block until the aortic specimen was on the end of the block. Excess epon was trimmed from the block and several two micron sections (referred to as a "thick" sections) were cut from the block and placed onto a glass slide. A drop of distilled water was placed over the specimens and the slide placed on a hot plate to fix the specimens to the slide. The slide was then stained with methylene blue and examined under a light microscope at 400 and 1000 times magnification. If a lesion was present, this area of the specimen was selected for further sectioning. If no lesion was identified, a random section of the specimen was selected. The block was then trimmed with a razor blade to form a pyramid with the selected area at the apex of the pyramid. Sections from the block (referred to as "thin" sections) were cut with a glass knife and floated onto a water bath. The thickness of the sections was identified by their color in the bath and silver to gold sections (60 - 90 nm) were preferred. Once a sufficient number of satisfactory specimens was obtained, the specimens were picked up onto a 300 mesh uncoated copper grid. The specimens were then air dried on

the grid.

The block was removed from the microtome and several millimeters, including the previous pyramid, were removed from the end of the block. The same procedure was then repeated a second time to procure a second slide with thick sections and a copper grid with thin sections. One further trimming of the block and sectioning was subsequently carried out. In total therefore, each block provided three slides with thick two micron sections and three copper grids with sixty to ninety nanometer corresponding thin sections from three separate random levels of the aortic specimen. This gave a total of ninety six slides with thick sections and ninety six copper grids with thin sections to examine (three samples from each of two blocks for the sixteen rats).

The thin sections were stained with uranyl acetate and lead citrate and were ready for observation in the transmission electron microscope.

Examination Techniques

The ninety six slides with thick sections were randomized using a random number chart generated by a computer and numbered sequentially from one to ninety six. The slides were examined by both the author and an experienced anatomical pathologist in a blinded fashion. The sections were graded as either normal or abnormal with note being made of the type of abnormalities present. Following this the code was broken and the results analyzed. The significance of variations in the incidence of lesion frequency among the two age groups and among the two phenotypes was determined using a chi squared analysis with one degree of freedom. Lesions identified in this manner were later

photographed for documentation and analysis.

The sixteen SEM samples were examined in a Philips SEM 505 scanning electron microscope (Philips, Eindhoven, Netherlands). The entire endothelial surface was examined and all areas of abnormal endothelium were recorded for assessment. Original magnifications (prior to magnification incurred in printing a picture from the negative) up to 2,500 times were used with an electron beam acceleration of 15kV.

The ninety six TEM samples were examined in a Philips TEM 410 transmission electron microscope (Philips, Eindhoven, Netherlands). Representative fields from each section in addition to lesions identified at the time of examination were photographed for analysis. Original magnifications of up to 26,000 times were used with an electron beam acceleration of 80 kV.

The major difficulty with TEM is differentiation of the type of cells present. One must take into account the location of the cell and the presence of various organelles in the cell. The three cell types that required differentiation were the endothelial cell, the macrophage and the smooth muscle cell. The differentiation becomes even more difficult when, as is the case in atherosclerosis, the cells are in an abnormal location or have been damaged. This is reflected by the current interest in monoclonal antibodies in an attempt to distinguish the smooth muscle cells and macrophages in arterial lesions.

Reidy and Schwartz have examined the ability to identify endothelium conclusively and noted there are several techniques by which to characterize these cells[453]. Endothelial cells have only two definite characteristics which separate them structurally from other cells. This is the presence of von Willebrand protein which can

be stained via immunofluorescence and the presence of Weibel-Palade bodies. Weibel and Palade first demonstrated these unique endothelial structures in lung arterioles in 1964[452]. Weibel-Palade bodies are small rod-shaped bodies which often occur in groups near the nucleus of the cell, commonly near mitochondria and the endoplasmic reticulum. They may be circular or elliptical, depending on the plane of section, and are bounded by a tight membrane. Centrally, they are occupied by tubules with the occasional presence of a central dense dot. Wagner, using immunofluorescence staining, has suggested that the Weibel-Palade bodies may be storage and/or processing organelles for von Willebrand protein[451]. The differentiation from mitochondria involves the fact that the mitochondria have a double layered membrane and cristae which are perpendicular to the outer surface, rather than tubules that run longitudinally.

Macrophages are characterized by a lobulated nucleus. The amount of cytoplasm is variable though lysosomes and vesicles with cellular debris (secondary lysosomes) are often identifiable in the cytoplasm. Ross also noted the presence of a distinct chromatin distribution in the nuclei which is quite distinct from that found in smooth muscle cells. The cells characteristically develop cellular extensions referred to as pseudopods which spread out into the surrounding tissue.

Smooth muscle cells on the other hand have a more regular, central nucleus. There are often small vesicles immediately underlying the cellular membrane, but no lysosomes. Within the cytoplasm are myofibrils which are usually poorly organized.

EXPERIMENTAL OBSERVATIONS

Light Microscopy

The ninety six slides with aortic sections were examined by the author and an anatomic pathologist. Prior to examining the slides, they were placed in a random order so that there was no chance that knowledge of the age and genotype of the animal would influence the decision as to whether an abnormality was present. The decision as to whether the section was normal or abnormal represented a consensus view between the two examiners. In the case where there was some uncertainty as to whether a lesion was present the slide was considered normal.

Prior to a description of the histology of the lesions observed by light microscopy, the quantitative aspect of the lesion distribution will first be presented. Suffice it to say at this stage that the majority of lesions were characterized by endothelial irregularity and not marked changes, though quite dramatic lesions were observed in seven of the ninety six specimens.

Overall, aortic sections which had abnormalities of their intima comprised 48% of all sections examined. When broken down by genotype, 56% of the corpulent (cp/cp) rat aortic sections were graded as abnormal whereas 40% of the thin (+/+) rat aortic sections were abnormal.

We will first consider the distribution of abnormal sections among the different animals by genotype. When the aortic specimens from both the six month and fourteen month old rats were combined into one group

and the presence of an abnormality was examined in relation to the genotype the following results were obtained:

Table 1. Distribution of abnormalities by genotype - all ages

GENOTYPE	NORMAL	ABNORMAL
+/+ (n=48)	60%	40%
cp/cp (n=48)	44%	56%
total (n=96)	52%	48%

$x^2 = 2.67$ $p = 0.102$

These statistics indicate that there is no significant relationship between the incidence of acrtic lesions among the corpulent and thin rats if differences in age are ignored. An analysis for each of the two age groups individually did demonstrate a relationship as follows:

Table 2. Distribution of abnormalities by genotype - 6 months

GENOTYPE	NORMAL	ABNORMAL
+/+ (n=24)	83%	17%
cp/cp (n=24)	50%	50%
total (n=48)	67%	33%

$x^2 = 6.00$ $p = 0.014$

Table 3. Distribution of abnormalities by genotype - 14 months

GENOTYPE	NORMAL	ABNORMAL
+/+ (n=24)	38%	62%
cp/cp (n=24)	38%	62%
total (n=48)	38%	62%

$x^2 = 0$ $p = 1$

It becomes apparent that although there is no overall difference there is a significant difference in the incidence of aortic lesions between fatty (cp/cp) and thin (+/+) animals at six months of age with this variation disappearing by fourteen months of age.

An alternate method of examining the data is to compare the incidence of lesions between the six and fourteen month old rats to see if there is an increase in the incidence of aortic lesions with age. Combining the two genotypes the results are as follows:

Table 4. Distribution of abnormalities by age - both genotypes

AGE	NORMAL	ABNORMAL
6 months (n=48)	67%	33%
14 months (n=48)	38%	62%
total (n=96)	52%	48%

$x^2 = 8.18$ $p = 0.004$

Among all animals, including both corpulent and thin, there is a very significant increase in the incidence of lesions with age. Data for the corpulent rats, analyzed separately, showed the following:

Table 5. Distribution of abnormalities by age - cp/cp

AGE	NORMAL	ABNORMAL
6 months (n=24)	50%	50%
14 months (n=24)	38%	62%
total (n=48)	44%	56%

$x^2 = 0.76$ $p = 0.383$

As one can see there is no significant increase in the frequency of aortic lesions among the corpulent rats as they age from six to fourteen months. However, a very significant increase occurs among the thin rats with age as the following data demonstrate:

Table 6. Distribution of abnormalities by age - +/+

AGE	NORMAL	ABNORMAL
6 months (n=24)	83%	17%
14 months (n=24)	38%	62%
total (n=48)	60%	40%

$x^2 = 10.54$ $p = 0.001$

As mentioned, the nature of the lesions which were observed varied from minimal endothelial irregularity to rather dramatic lesions with a proliferative lesion extending into the media of the arterial wall. The normal endothelium was characterized by a smooth, single-celled layer without any subendothelial irregularity. Examples of a normal endothelium are provided in Plates 1 and 2. Note the smooth laminations of the media underlying the endothelium.

Plates 3, 4 and 5 demonstrate the most common type of endothelial lesion observed. The endothelium is characterized by an irregularity with a saw-toothed type of irregularity. There is proliferation of the endothelium in locations as well as accumulation of subendothelial debris. These findings subjectively appeared to be more severe and gross in the fourteen month old corpulent animals when the slides were reviewed a second time.

More typical and definitive atherosclerotic lesions occurred in a total of seven specimens. Of this small group of lesions, one was found in a six month corpulent rat, two were found in fourteen month lean rats and the remaining four were found in fourteen month corpulent rats. There was only one instance where the same rat provided two severe lesions among the three sections from each rat in the case of one of the fatty fourteen month old rats. The remainder of the lesions all came from separate animals.

The one atherosclerotic lesion identified in six month old animals occurred in a corpulent animal and the lesion is presented in Plate 6. The internal elastic lamina is deficient underlying the cellular lesion.

The two lesions identified in the fourteen month lean animals are

PLATE 1. Light microscopy - 6 month +/+
Normal endothelium. Magnification 1250 times (bar = 10 um). Methylene blue staining.

PLATE 2. Light microscopy - 6 month cp/cp
Normal endothelium. Magnification 1250 times (bar = 10 um). Methylene blue staining.

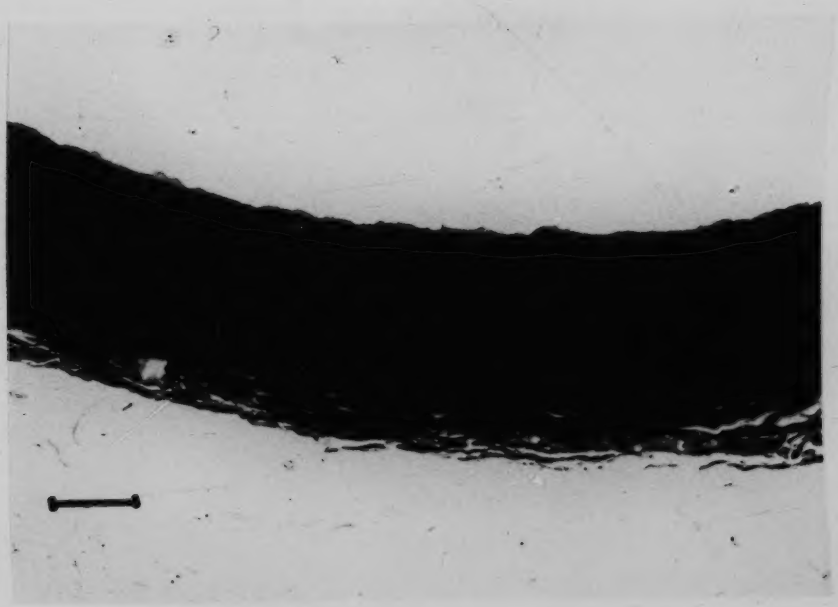

PLATE 3. Light microscopy - 14 month +/+
Abnormal endothelium with irregularities of the surface in a saw-tooth type of pattern. Magnification 1250 times (bar = 10 um). Methylene blue staining.

PLATE 4. Light microscopy - 14 month +/+
Abnormal endothelium with a more prominent irregular endothelium. Magnification 1250 times (bar = 10 um). Methylene blue staining.

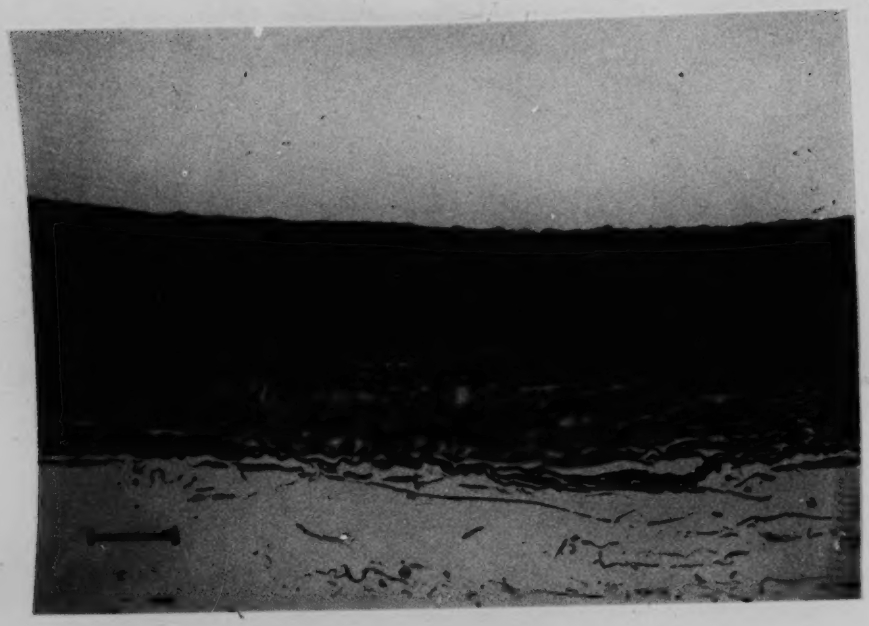

PLATE 5. Light microscopy - 14 month +/+
Abnormal endothelium. Magnification 1250 times (bar = 10 um).
Methylene blue staining.

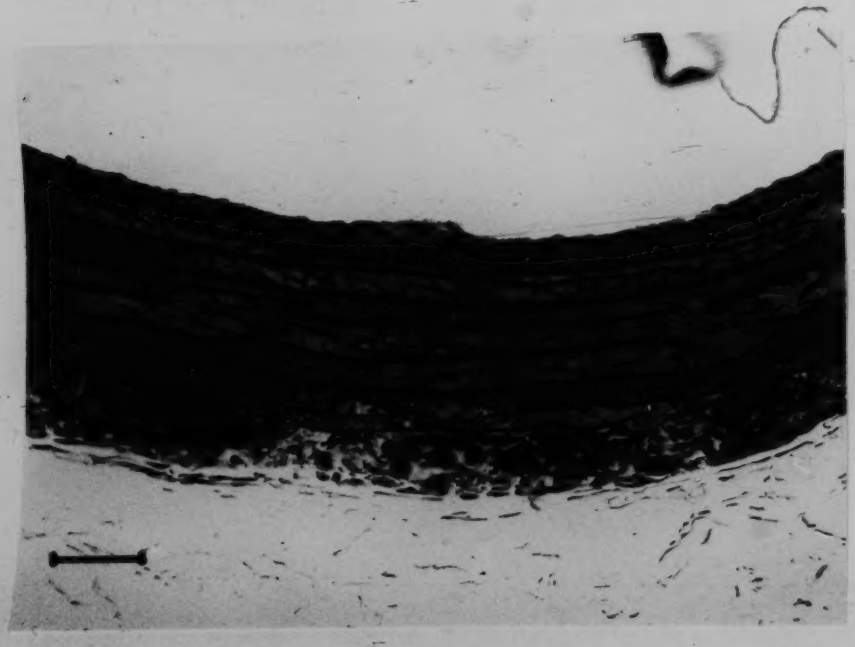

PLATE 6. Light microscopy - 6 month cp/cp
Cellular lesion which has eroded through the internal elastic lamina.
Magnification 1250 times (bar = 10 um). Methylene blue staining.

presented in Plates 7 and 8. Both are proliferative and the lesion in Plate 8 shows degenerative elements in the lesion plus lighter staining material which may represent lipid material.

The four lesions identified in the fourteen month corpulent animals are presented in plates 9 through 12. In Plate 10, the lesion is invading through the internal elastic lamina into the underlying media. This is demonstrated near the left side of the Plate.

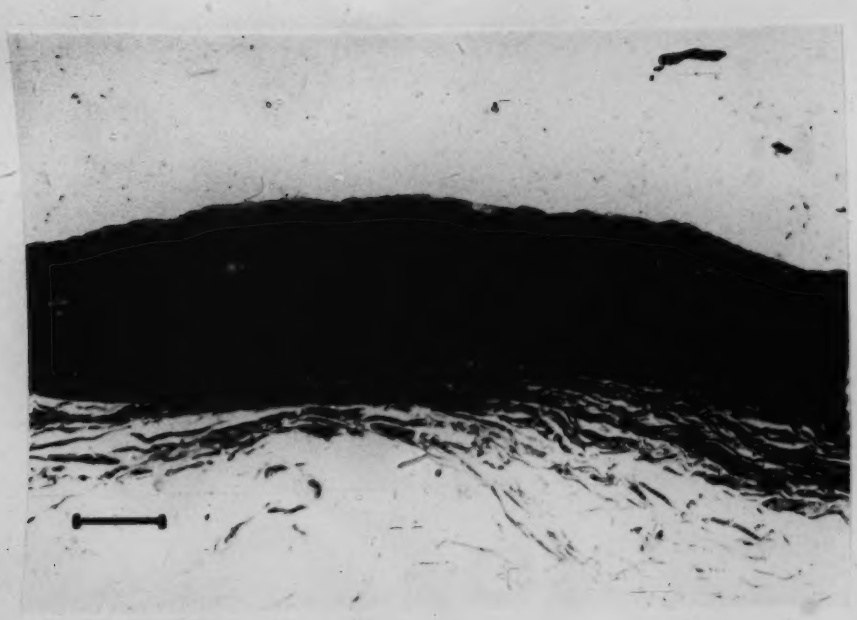

PLATE 7. Light microscopy - 14 month +/+
Very prominent endothelial thickening and loss of endothelial monolayer. Magnification 1250 times (bar = 10 um). Methylene blue staining.

PLATE 8. Light microscopy - 14 month +/+
Large raised intimal lesion which contains lipid and debris. Magnification 1250 times (bar = 10 um). Methylene blue staining.

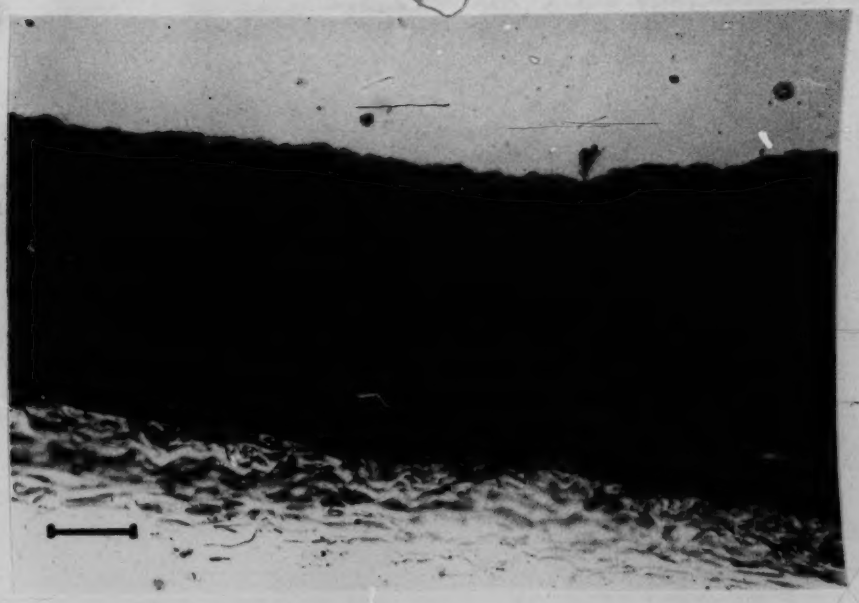

PLATE 9. Light microscopy - 14 month cp/cp
Diffuse intimal thickening and irregularity. Magnification 1250 times (bar = 10 um). Methylene blue staining.

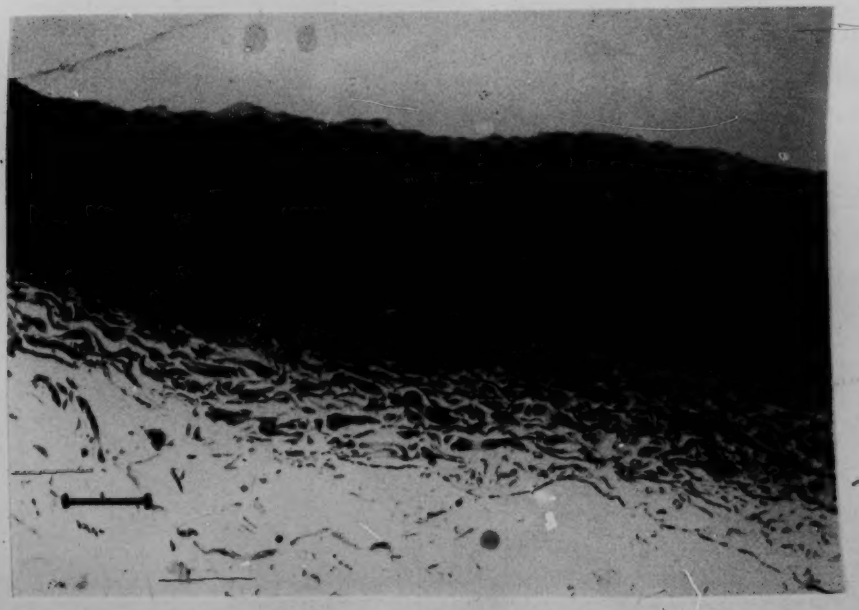

PLATE 10. Light microscopy - 14 month cp/cp
Intimal lesion. Magnification 1250 times (bar = 10 um). Methylene blue staining.

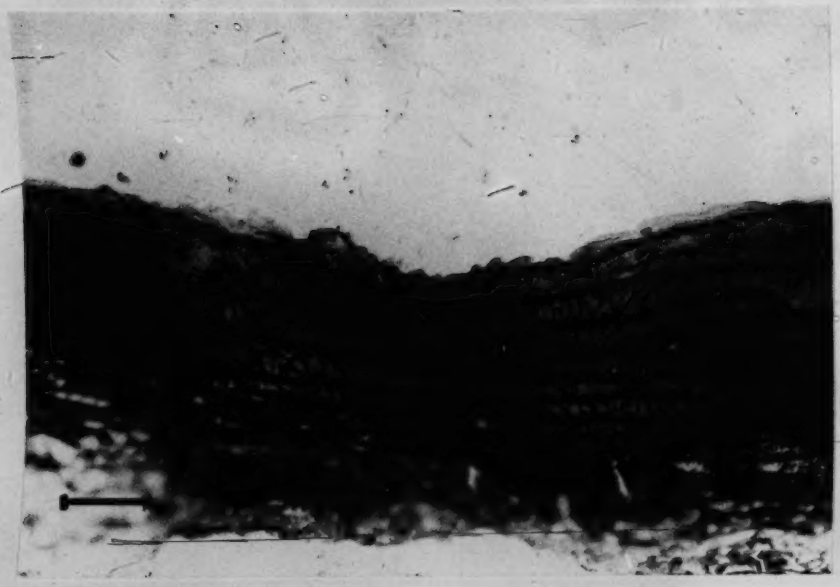

PLATE 11. Light microscopy - 14 month cp/cp
Proliferative endothelial lesion. Magnification 1250 times (bar = 10 um). Methylene blue staining.

PLATE 12. Light microscopy - 14 month cp/cp
Proliferative endothelial lesion. Magnification 1250 times (bar = 10 um). Methylene blue staining.

Scanning Electron Microscopy

A total of sixteen aortic segments were examined by scanning electron microscopy. This included four specimens from each of the four groups - six month leans, six month corpulents, fourteen month leans and fourteen month corpulents. A low power magnification of a normal endothelium is presented in Plate 13. This shows the orientation of the sections examined which represent one half of the aortic arch of each rat. The proximal ostia of one of the arch branches can be seen towards the left of the micrograph. Note the undulations of the surface which are felt to reflect contractions of the underlying elastic tissue. A higher magnification of the surface in Plate 13 is presented in Plate 14 and the monotonous pattern of endothelial cells with their prominent nuclei can be seen. The appearance of the raised nuclei was seen very commonly.

In the six month animals, no lesions were demonstrated in the lean (+/+) rats and only one of the four corpulent (cp/cp) rats demonstrated any significant lesions. Plate 15 shows the areas of cellular and nuclear dropout which were observed in this animal. However, the remaining cells retain their marginal folds and semitrapezoid shape. It was from the aortic section of this same animal that the thick section shown in Plate 2 and the thin section shown in Plate 25 were obtained.

The endothelium of the fourteen month old thin (+/+) rats was not as regular and well maintained as that in the six month old rats. Similar lesions to those in the six month rats, characterized by deendothelialization and missing nuclei, were present (Plate 16).

PLATE 13. SEM - 6 month +/+
A low magnification view of the entire surface. Surface undulations are prominent. Magnification 215 times (bar = 50 um). Sputter coated with gold.

PLATE 14. SEM - 6 month +/+
A higher magnification of a normal endothelium. Note the monotonous pattern of the nuclei which are slightly elevated above the cytoplasm. Magnification 1560 times (bar = 10 um). Sputter coated with gold.

PLATE 15. SEM - 6 month cp/cp
The outline of the cells is clearly visible. Several cells have dropped out and nuclear dropout alone of two cells with adherent material is seen in the upper corners. Magnification 6250 times (bar = 2 um). Sputter coated with gold.

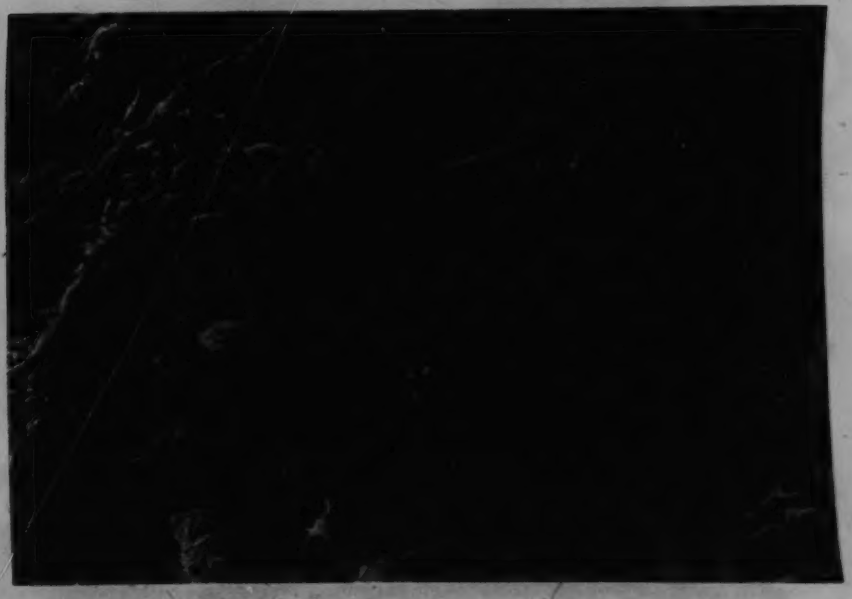

PLATE 16. SEM - 14 month +/+
Similar lesions to those shown in Plate 15 in an old thin rat with several areas of denudation and exposure of the underlying subendothelium. The remaining endothelium is not well preserved. Magnification 6550 times (bar = 2 um). Sputter coated with gold.

However, note that unlike the lesions in the six month corpulent rat (Plate 15), the remaining endothelium is not well maintained. Also present in the fourteen month thin rats are heaped up lesions and pyramidal cells (Plate 17). Both Plates 16 and 17 were obtained from the same rat. The thin sections in Plates 34, 35 and 36 were obtained from the corresponding aortic segment used for TEM from this same rat.

The fourteen month old corpulent (cp/cp) rats showed another gradation up in the frequency and type of lesions observed. Also present were the pyramidal cells and heaped up lesions (Plate 18). However, associated with these lesions were more prominent surface irregularities with deendothelialization and exposure of the underlying subendothelium. Plate 19 is a higher magnification of the surface of Plate 18 demonstrating these changes. A similar type of lesion in another of the corpulent fourteen month old rats is presented in Plate 20.

Plate 21 shows a very prominent plaque observed on the aorta of a third rat in the fourteen month corpulent group. Plate 22 shows a very high magnification of the surface of this lesion.

The corresponding aortic segments to those from which Plates 18 and 19 were obtained provided the thin sections for Plates 39 and 40 while Plates 21 and 22 similarly correspond with the thick section presented in Plate 9.

PLATE 17. SEM - 14 month +/+
Heaped up lesions with several pyramidal cells. Magnification 815 times (bar = 10 um). Sputter coated with gold.

PLATE 18. SEM - 14 month cp/cp
Elevated lesions with loss of endothelial cells on the surface of the lesions. Magnification 1560 times (bar = 10 um). Sputter coated with gold.

PLATE 19. SEM - 14 month cp/cp
Higher magnification of the heaped up lesion in Plate 18. Not only is there loss of complete endothelial cells, but one relatively intact cell without its nucleus is present in the right center of the picture. Magnification 6250 times (bar = 2 um). Sputter coated with gold.

PLATE 20. SEM - 14 month cp/cp
Another old fatty with significant surface irregularities and denudation with loss of cover of the underlying subendothelium. Magnification 3275 times (bar = 2 um). Sputter coated with gold.

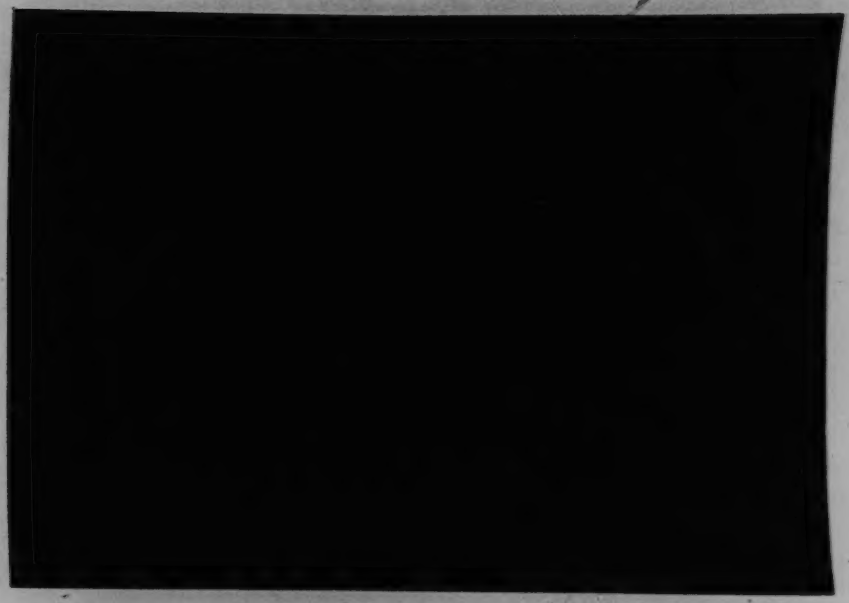

PLATE 21. SEM - 14 month cp/cp
A large prominent lesion on the aortic surface. Magnification 680 times (bar = 10 um). Sputter coated with gold.

PLATE 22. SEM - 14 month cp/cp
High magnification of the surface of the lesion of Plate 21 showing an area of cell denudation. Magnification 27250 times (bar = 0.5 um). Sputter coated with gold.

Transmission Electron Microscopy

A total of ninety six copper grids with thin sections were examined. This comprised an examination of six different levels of the aortic arches of the four rats in each of the four groups.

The six month thin (+/+) rats did not show a high incidence of abnormalities. A normal endothelium is shown in Plate 23 from a lean six month rat. Note the endothelial monolayer with multiple pinocytic vesicles. The internal elastic lamina can be seen underlying the endothelium with the edge of an underlying smooth muscle cell visible beneath. Note also the presence of several Weibel-Palade bodies in the surface endothelium - the only characteristic structural feature of endothelial cells.

Several small lesions, such as that shown in Plate 24, were identified in the six month thin rats. However, these lesions were small and predominately composed of vacuoles in the endothelium. Some of these vacuoles, such as the two shown in this plate, had fused with the surface to release their contents. The debris which is seen intervening between the endothelium and internal elastic lamina in this plate was a common finding near these vacuoles.

The six month corpulent (cp/cp) animals show a wider variety of abnormalities. The vacuoles noted previously were again observed (Plates 25 and 26). An increasing amount of debris and lipid was present near these vacuoles and in surrounding areas. In addition to the debris, cells not normally present in the subendothelial space also appeared between the endothelium and the internal elastic lamina. Some of the cells appeared to be macrophages (Plate 25) with pseudopods of

PLATE 23. TEM - 6 month +/+
Normal endothelium. Note the pinocytic vesicles and the internal elastic lamina. Several Weibel-Palade bodies are present in the endothelium. Corresponding thick section in Plate 1. Magnification 28600 times (bar = 0.5 um). Uranyl and lead staining.

PLATE 24. TEM - 6 month +/+
Larger vacuoles appearing to contain debris fusing with the surface. Subendothelial debris also present. The internal elastic lamina is seen at the base of the picture. Magnification 30800 times (bar = 0.5 um). Uranyl and lead staining.

PLATE 25. TEM - 6 month cp/cp
Vacuoles with debris near the nucleus of an endothelial cell. Macrophages are present in the subendothelial space above the internal elastic lamina in addition to lipid and cellular debris. Magnification 12600 times (bar = 1 um). Uranyl and lead staining.

PLATE 26. TEM - 6 month cp/cp
Subendothelial smooth muscle cell and lipid. Vacuoles are present in the overlying endothelium. The internal elastic lamina can be seen at the base of the photograph. Corresponding thick section in Plate 2. Magnification 22750 times (bar = 0.5 um). Uranyl and lead staining.

the cell extending into the surrounding tissue and no contractile elements in the cytoplasm. Some appeared to more closely resemble smooth muscle cells with a more regular nuclear outline, and myofibrils within the cytoplasm (Plate 26).

One further feature observed in the six month corpulent animals was the presence of cells traversing the internal elastic lamina. Plates 27 and 28 show two different magnifications of what appears to be a process of the underlying smooth muscle cell going through the elastic lamina and communicating with the subendothelial space. Plates 29 and 30 show an entire cell disrupting the integrity of the internal elastic lamina. This cell appears to be a macrophage, rather than a smooth muscle cell, as pseudopods are present in the cytoplasm in addition to a lobulated nucleus. In both these examples there is associated debris present in the subendothelial space.

In the fourteen month old thin (+/+) rats, the lesions were much larger than those in the both the fat and thin six month old animals. Plates 31 and 32 show two different lesions with large amounts of lipid. In Plate 32, the internal elastic lamina is discontinuous underlying the subendothelial accumulation of material with apparent continuity between the subendothelial space and the underlying media.

Debris laden vacuoles were also identified in the subendothelial area of aortas from the fourteen month lean rats, as shown in Plate 33. Note in this plate the prominent Weibel-Palade bodies in the endothelial cell.

Plates 34, 35 and 36 are different magnifications of an interesting lesion observed in one of the fourteen month thin (+/+) rats. There are two compressed endothelial cells on either side of an abnormal cell

PLATE 27. TEM - 6 month cp/cp
A pseudopod of a smooth muscle cell protrudes through the internal elastic lamina into the subendothelial space. Magnification 24500 times (bar = 0.5 um). Uranyl and lead staining.

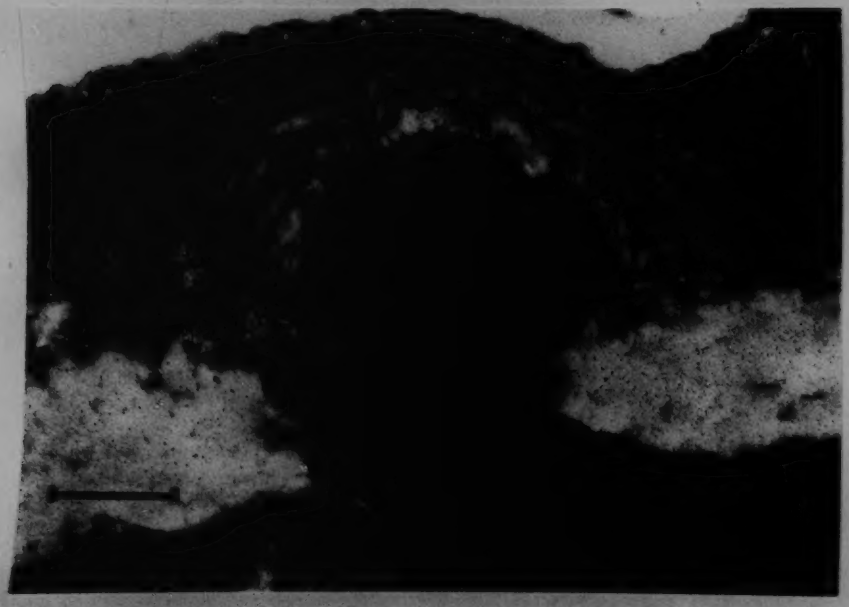

PLATE 28. TEM - 6 month cp/cp
Higher magnification of the lesion in Plate 27. Magnification 36500 times (bar = 0.5 um). Uranyl and lead staining.

PLATE 29. TEM - 6 month cp/cp
A macrophage is penetrating through the internal elastic lamina. Magnification 7800 times (bar = 1 um). Uranyl and lead staining.

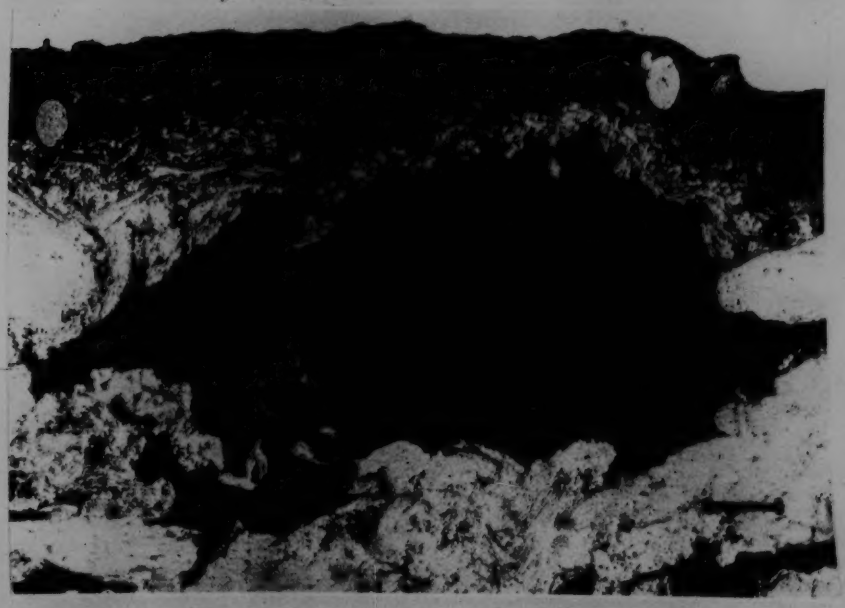

PLATE 30. TEM - 6 month cp/cp
Higher magnification of Plate 29. Magnification 10725 times (bar = 1 um). Uranyl and lead staining.

PLATE 31. TEM - 14 month +/+
Subendothelial lipid and debris with overlying surface irregularity and condensation of the endothelial nucleus. Corresponding thick section in Plate 7. Magnification 26000 times (bar = 0.5 um). Uranyl and lead staining.

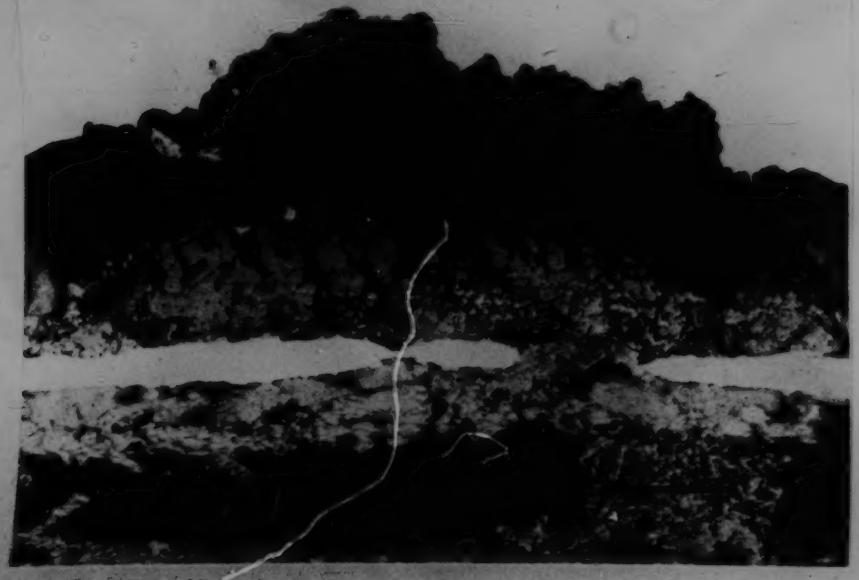

PLATE 32. TEM - 14 month +/+
Another lesion from the same rat as Plate 31. Note the transgression of the internal elastic lamina by debris. Magnification 17500 times (bar = 1 um). Uranyl and lead staining.

PLATE 33. TEM - 14 month +/+
A vacuole immediately underlying the nucleus of an endothelial cell. Note the prominent Weibel-Palade bodies. Corresponding thick section in Plate 3. Magnification 26000 times (bar = 0.5 um). Uranyl and lead staining.

PLATE 34. TEM - 14 month +/+
Two compressed endothelial cells surrounding an abnormal cell which is immediately below the endothelium. The endothelium is discontinuous over this cell near the T shaped cellular fragment. Magnification 9100 times (bar = 1 um). Uranyl and lead staining.

PLATE 35. TEM - 14 month +/+
Higher magnification of Plate 34. Several Weibel-Palade bodies in the abnormal cell in addition to several mitochondria suggesting it is an endothelial cell. Magnification 13650 times (bar = 1 um). Uranyl and lead staining.

PLATE 36. TEM - 14 month +/+
A higher magnification of Plate 34. Note the heaped up nucleus of the endothelial cell and the loss of cover near the T shaped cellular fragment. Magnification 22000 times (bar = 0.5 um). Uranyl and lead staining.

which underlies the endothelium. This cell has mitochondria present as well as two Weibel-Palade bodies, best seen in Plate 35, suggesting that it is an endothelial cell. There is associated discontinuity of the internal elastic lamina underlying this cell. The endothelium is discontinuous near the T shaped piece of cellular debris.

One further observation of endothelial irregularity was made in aortic sections from the fourteen month old thin rats. In Plate 37, the nucleus of an endothelial cell is protruding into the arterial lumen because of a subendothelial accumulation of lipid and debris. An associated abnormality of the adjoining endothelium has produced a pseudovillous pattern. This may correspond to the pseudovillous pattern sometimes seen on SEM examination of the aortic surface.

A similar pseudovillous pattern was present to a greater degree in the fourteen month old corpulent (cp/cp) rats as demonstrated in Plate 38. Again the accumulations of subendothelial debris and vacuoles as seen in the other rats was also present in these rats. Subendothelial cellular elements were again identified. The subendothelial cells in Plates 39 and 40 are consistent with smooth muscle cells with disorganized myofibrils and central nuclei.

The lesion shown in Plate 41 appears to resemble the type of lesion identified in Plates 34, 35 and 36. The cell has Weibel-Palade bodies in the cytoplasm and can best be characterized as an abnormal endothelial cell in an area of grossly deranged endothelium.

Plates 42, 43 and 44 demonstrate the binding of monocytes to the cell surfaces with apparent subendothelial migration. Each of these three cells shows a large nucleus and many mitochondria suggesting the

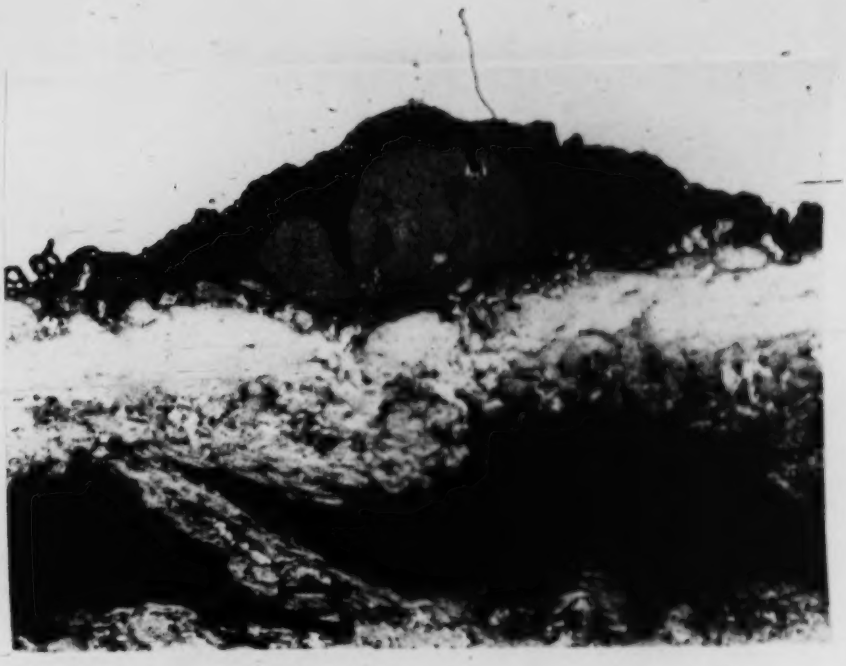

PLATE 37. TEM - 14 month +/+
Subendothelial lesion and irregularity of internal elastic lamina. Pseudovillous pattern of endothelium to the left of the nucleus. Magnification 9900 times (bar = 1 um). Uranyl and lead staining.

PLATE 38. TEM - 14 month cp/cp
Marked irregularity with pseudovilli of the endothelium overlying a collection of lipid and debris. Corresponding thick section in Plate 10. Magnification 8250 times (bar = 1 um). Uranyl and lead staining.

PLATE 39. TEN - 14 month cp/cp
High magnification of a subendothelial smooth muscle cell. Debris in the area is arranged concentrically around the cell. Magnification 29700 times (bar = 0.5 um). Uranyl and lead staining.

PLATE 40. TEN - 14 month cp/cp
A large smooth muscle cell immediately underlying the endothelium. Magnification 29700 times (bar = 0.5 um). Uranyl and lead staining.

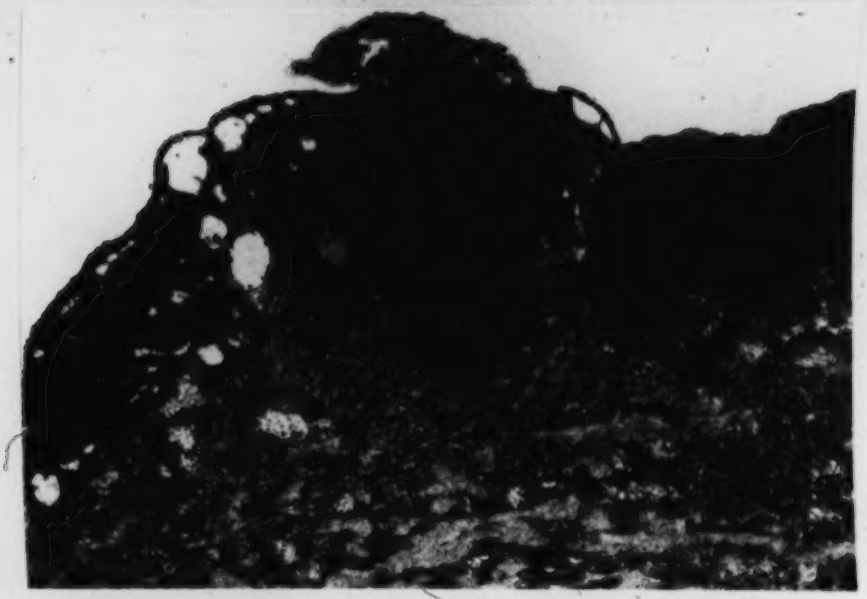

PLATE 41. TEM - 14 month cp/cp
An abnormal endothelial cell with Weibel-Palade bodies underlying a grossly abnormal endothelium. Magnification 10500 times (bar = 1 um). Uranyl and lead staining.

PLATE 42. TEM - 14 month cp/cp
A macrophage attached to a grossly abnormal surface and penetrating the endothelium near a cleft. Note the pseudopods of the penetrating cell and debris in the apex of the cell. Magnification 14000 times (bar = 1 um). Uranyl and lead staining.

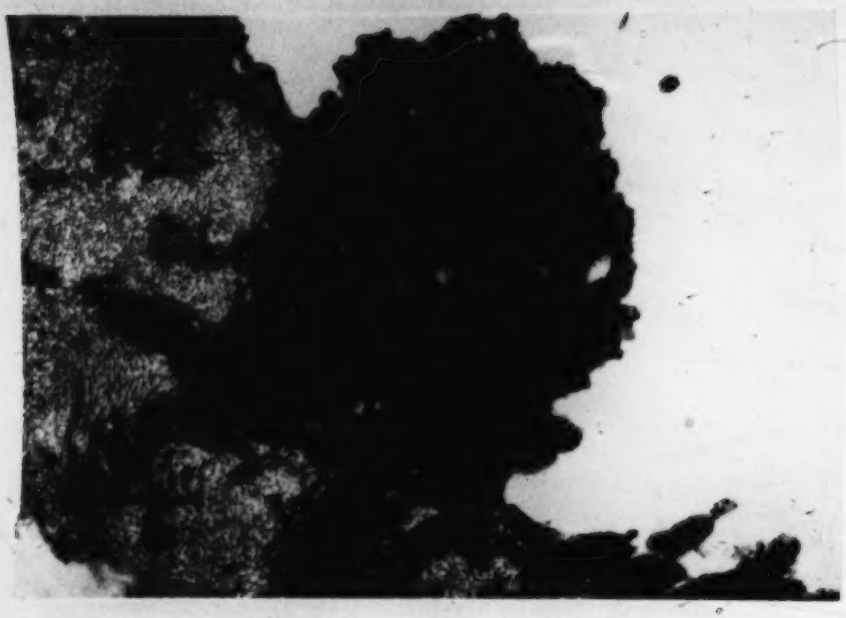

PLATE 43. TEM - 14 month cp/cp
A macrophage attached to the surface with pseudopods penetrating into the subendothelial space. Magnification 18000 times (bar = 1 um). Uranyl and lead staining.

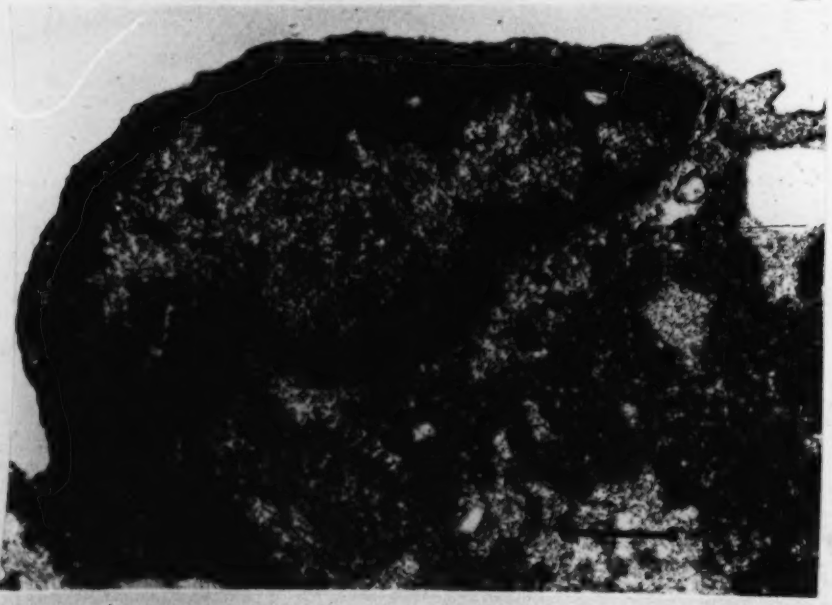

PLATE 44. TEM - 14 month cp/cp
Macrophage attached to the surface. Magnification 30800 times (bar = 0.5 um). Uranyl and lead staining.

cells are still metabolically active. There are extensions (pseudopods) from these cells which invade into the underlying tissue. In addition to the accompanying subendothelial lesions, the overlying and adjacent endothelium is markedly abnormal.

DISCUSSION AND CONCLUSIONS

I would first like to summarize and examine the data obtained from each aspect of this experiment. An attempt to account for the observed explanations will be presented followed by a discussion of the three questions posed at the conclusion of the literature review to see if they have been answered.

A Little Summarizing

By light microscopy, it is evident that significant vascular lesions do indeed occur in the LA/N-cp rat. The corpulent (cp/cp) rat appears to develop lesions at an earlier age than the lean (+/+) rat. The significant difference in the incidence of lesions at six months of age disappears by fourteen months of age. This would seem to indicate that the corpulent rats develop premature atherosclerotic disease with atherosclerosis occurring in both lean and corpulent animals with age. One could postulate that the premature atherosclerosis may result from the hyperlipidemia present in the corpulent rats, as appears to be the case in human disease. The difference at six months could just as easily be due to the hyperinsulinemia or even more likely a combination of these two factors. There are other possible etiologic factors which are involved but still not accounted for which could lead to the premature atherosclerosis in this animal model.

The significant prematurity of the lesions in the corpulent rats, for whatever reason, is potentially very useful. If, for example, one wanted to prove that a given drug significantly decreases the incidence of premature atherosclerosis in man, this animal model may be ideal. If the

addition of the drug to a group of corpulent rats decreases the incidence of lesions so that at six months there is no significant difference in the incidence of lesions between corpulent rats and age matched lean rats, this would be good evidence of a positive effect of the drug. Because the rats are moderately inexpensive, it would be easy to add a control thin group which could receive the drug to demonstrate the absence of significant side effects as well as a second control group of corpulent rats not receiving the drug. The results from such an experiment could be available within a reasonably short six month time period.

The fact that the corpulent rat develops premature atherosclerosis is akin to the human condition. All humans will develop atherosclerosis if they live long enough and it is a conceded fact that atherosclerosis occurs as part of the "natural aging" phenomena. The area of greatest concern centers around forms of prevention for the premature death from atherosclerosis secondary to known risk factors in the population. The moderate endogenous increase in cholesterol present in this animal (which resembles human Type IV hyperlipidemia) makes the model ideal for manipulations of serum cholesterol and the effect this has on the incidence of atherosclerosis.

An examination of the histology of the lesions demonstrated by light microscopy shows a wide variety of changes in the LA/N-cp rat. The atherosclerotic process appears to start at an early age with significant lesions present in six month old homozygous recessive rats (cp/cp) but not the lean (+/+) animals. There is an associated gradation with age as the more severe lesions occur in the older animals for both genotypes.

The scanning electron microscope has recently become a very popular method of examining arterial surfaces for cell damage primarily as a

result of its resolution and ability to easily examine large surfaces. Its greatest power is in the detection of endothelial cell loss with resultant exposure of the underlying subendothelium. The major stumbling block has been that it is difficult to correlate the findings observed on SEM with the more standard sectional view of arteries as examined under the light and transmission electron microscope. SEM also tends to underestimate the degree of vessel damage. It has been shown that small areas of endothelial loss in vivo can be rapidly repaired by migration of surrounding endothelial cells without any abnormality of the surface being detectable[453]. As well, smooth muscle cells have the capability of forming a pseudo-endothelium following injury which can very closely resemble endothelial cells and be regarded as normal.

Accepting these limitations, SEM has been used extensively to examine arterial surfaces. The changes in the LA/N-cp rat have been well characterized by Russell and Amy[444]. The same changes observed in other animal models have been documented to occur in this animal model. The proposed progression of lesions starts with raised nuclei and progresses to the adherence of macrophages on the surface. Loss of the nucleus and eventually the entire cell follow. It is the healing of these areas with the potential for the incorporation of macrophages and platelets which is postulated to result in the appearance of the polygonal cells. Further endothelial damage would then lead to progression of the lesions.

Though this proposed progression has not yet been verified with the direct TEM examination of the raised, advanced lesions observed by SEM, it would appear by examination of the SEM micrographs obtained in the LA/N-cp rat that this is the natural progression of lesions. Raised nuclei appear to be a common finding in normal cells. The first lesion

observed, in the younger less affected lean animals, is the appearance of nuclear and cellular loss. The remaining endothelium becomes progressively more damaged with age and it is only in the older rats that the raised and polygonal lesions appear. An increased incidence of all lesions appears to develop in the corpulent animals in both age groups. This would indicate that not only do the corpulent animals get a premature form of atherosclerosis but the disease they get is more severe than age matched lean rats.

The one thing not observed in this study, in relation to the proposed progression of lesions, is the adherence of macrophages to the arterial surface. As this has been previously well documented to occur in the IA/N-cp rats, this may be the result of an insufficient number of specimens.

By far the most interesting observations were made by TEM examination of the arteries. There were many different lesions observed in the specimens examined. The most frequent lesions were the raised lesions with what I refer to as debris. The debris consists of many different cellular constituents. Collagen, elastic tissue and intracellular matrix are all present. As well, in some sections, there appeared to be a laminated appearance of debris which had the appearance of tubules or myofibrils around several of the subendothelial cells which were present. The cellular constituents which were present in the subendothelial layer included smooth muscle cells, macrophages and abnormal endothelial cells.

The lesion which appeared first, in the younger lean rats, and appeared to be most frequent overall, was the presence of large vacuoles in the endothelial cells. These vacuoles were commonly found in the

subnuclear location and they appeared to eventually fuse with the cell membrane to release their contents. In this fashion they appeared to resemble secondary lysosomes. The vacuoles could be a reflection of endothelial damage with attendant swelling and degeneration. Alternatively, these vacuoles could represent the accumulation of cholesterol or VLDL.

An abnormality of the internal elastic lamina appeared to be a common phenomenon underlying subendothelial lesions. This may very well be secondary to chemotaxins produced in the lesions. Both macrophages and smooth muscle cells were demonstrated to interrupt the continuity of the internal elastic lamina.

Two types of observed lesions raise the most questions. The first is the subendothelialization of abnormal endothelial cells as shown in both Plate 34 and 41. In these two examples the abnormal endothelial cell is surrounded by the compressed nuclei of two adjacent endothelial cells. There does not appear to be any pseudopod formation by either of these two cells to suggest that they are macrophages engulfing dead endothelial cells. There are several mitochondria in these abnormal cells near the Weibel-Palade bodies suggesting that the cells are still metabolically active.

One explanation of this observation is that the abnormal cells have been severely damaged but not killed. The surface abnormality which results from the injury may be covered by the adjacent endothelial cells which migrate to the area. The usual supposition is that dead or injured cells desquamate and leave areas of exposed subendothelium as observed by SEM. However, this may occur only for the most severely injured cells while those which sustain a sublethal injury remain in place. With time,

this progressive encroachment of surrounding endothelial cells could lead to the observed relegation of the injured endothelial cell to the subendothelial location. Even though the cell may be injured, it could still produce many of the factors responsible for chemotaxis, leading to the attraction of other cells and being responsible itself for some of the debris.

The second possibility is that the cell may have undergone some form of transformation. It may actively move to a subendothelial position secondary to some type of injury or chemotactic factor and remain metabolically active. This would explain the large number of mitochondria observed in the two cells of Plates 34 and 41 which lead one to believe that the cells are still metabolically very active. This transformed cell would have even greater potential for causing the accumulation of other cells in the same area.

The second very interesting observation is demonstrated in Plates 42, 43 and 44. Macrophages are adherent to the surface and appear to be penetrating into the subendothelial space. The endothelium appears deficient in these areas. This phenomena was observed only in the old corpulent animals suggesting that it is a late occurrence in the development of the lesions. A large amount of subendothelial debris is already present and there are surrounding gross abnormalities of the endothelium.

These observations appear to contradict the response to injury hypothesis which proposes that the subendothelialization of macrophages is an early phenomena which precedes the development of an atherosclerotic lesion rather than following and magnifying it[27]. In the LA/N-cp rat this invasion by macrophages is seen to occur late. The early lesion in

this model appears to be the subendothelial migration of endothelial cells.

A Little Supposing

It may be that the sequence of events begins with endothelial damage of some sort. In an attempt to repair the damage, the injured endothelial cell may become metabolically transformed. In repairing the damage, the garbage from the "clean up" is dumped into vacuoles which leave the nuclear area to eventually fuse with the cell membrane. The fusion of these vesicles with the membrane could leave minute areas of exposed subendothelium which are quickly covered by adjacent cells. The cell may be unable to repair itself before the surrounding cells migrate to cover the defect secondary to the damage it sustained. This process could be repeated until such time as either the cell dies and is replaced or until such time as the surrounding cells completely overlap the endothelial cell. This would certainly be in keeping with the existance of endothelial stomata previously identified in the literature[453]. Explanations for these small circular structures which are an integral part of the endothelium but definitely not cells has ranged through suggestions that they constitute intracellular cement[454], groups of platelets[455], micro valves[456] and cell hernias[457]. The most recent explanation, in keeping with the theory being proposed, is that they are membrane flaps of adjacent cells[458,459]. It has already been noted that these stomata are related to cell injury and replication.

As the cell is not dead, but only injured, it will continue to survive in the subendothelial area although in a different microenvironment than it was accustomed to. Endothelial cells have been demonstrated to be capable of secreting numerous cell products including very potent

chemotaxins. The products of the cell could accumulate in the subendothelial area to add to the debris which is observed to accumulate. The chemotaxins produced could result in the migration of both smooth muscle cells and macrophages to the area resulting in the fully developed lesion.

In order to incorporate the SEM observations in this hypothesis, one would have to propose that the areas of endothelial and nuclear dropout result from those cells which are so severely damaged that they simply die and are swept away by the blood stream. With this argument, areas so affected with a denuding injury would not be the precursors to lesions but only damaged areas which had still not been repaired. One would expect a higher incidence of this type of lesion in circumstances where there is a great deal of endothelial damage. However, rather than the most damaged, and consequentially killed, endothelial cells causing the lesions it may be that the intermediately damaged cells are responsible for the bulk of the atherosclerotic lesions observed. The cell loss observed over the more advanced, protruding lesions would be by a different mechanism secondary to the underlying damage, ulceration, and loss of cover. Sufficient lesions are observed on TEM to account for the raised lesions observed on SEM.

The reason that the incidence of lesions more closely parallels the insulin resistance than it does the degree of hypercholesterolemia may be that the insulin resistance serves as a marker for a more global defect in the ability of the cell to repair itself. For the same reason that the cell does not respond to insulin by taking up the glucose from the bloodstream, it may not respond to cellular damage by synthesizing sufficient membrane to repair a defect before adjacent cells cover the

defect, especially if the defect is small. Thus in the presence of a more normal insulin response the cells may exhibit a more normal repair response.

The hyperlipidemia with an increased VLDL may be caused by either overproduction or inadequate clearance or a combination of both. One might suspect that it is due to inadequate clearance by the cells reflecting the inherent cellular damage already postulated. However, it is just as likely secondary to overproduction in response to a cellular messenger which is signalling the endothelial damage. The possibility that this messenger substance may be a component of the vacuoles which are observed to fuse with the membrane is an interesting possibility.

A Little Answering

Finally, I would like to return to the three questions proposed at the beginning of this project and see if they have been answered satisfactorily.

The first question was a query as to whether the lesions of the University of Alberta LA/N-cp rats were similar histologically to human atherosclerotic lesions. On this account, one must note that the lesions did demonstrate lipid and cellular debris. They were cellular, with both macrophages and smooth muscle cells present. No foam cells were observed and no proliferative fibrous caps were observed in any of the lesions. This may reflect a low number of observations and will require further elucidation.

The second question was whether or not there was any evidence for macrophage subendothelialization. The answer is a very grey yes. There was definite evidence of macrophages present in the subendothelial space and attached to the endothelium. However, this appeared to be a late

finding rather than a predisposing phenomena. Of even greater interest were the endothelial cells observed to be penetrating the endothelium.

The third question was a search for any evidence of progression of lesions. As stated earlier, there is excellent evidence for progression of lesions in the thin rats. The premature atherosclerosis of the corpulent rats did not allow for any significant progression in incidence with age though there is a subjective increase in severity.

A Little Concluding

I do not think that there is any doubt that the best animal model in terms of nature of the lesions for atherosclerosis is the noncholesterol fed primate. Its closeness to man phylogenetically is evidenced by the similarity of the lipoproteins and lesion histology. It will develop lesions naturally though at an advanced age and with a low incidence.

The major difficulty is posed by the circumstances of an experimental protocol. If one compares the cost of a monkey model and the LA/N-cp rat model, the difference in doing experiments is overwhelming. At the University of Alberta, the current cost of a obtaining a monkey is $425.00, maintenance charges are $3.00 per day and the cost of a major operation such as perfusion fixation is $60.00. The corresponding costs for the LA/N-cp rat are $30.00, $0.11 and $7.00. If we assume that, like the LA/N-cp rat, the monkey will produce a high incidence of lesions in six months time, the comparative cost of doing the procedure on one animal is $1025.00 for the monkey and $57.00 for the rat - an eighteen fold difference. When dealing with sufficient numbers of animals for the results of a study to be statistically significant, the difference is overwhelming. As well, I would have serious doubts about the assumption that the monkeys will develop a high incidence of lesions by six months of

age on a normal diet as the LA/N-cp rats will.

It may be possible to produce lesions in the monkey and other animal models with dietary cholesterol supplementation but there is abundant literature available indicating that cholesterol in the exogenous pathway may have different effects on the nature of the disease in the animal models than endogenous hypercholesterolemia. The LA/N-cp rat is unique in that it develops a high incidence of lesions without cholesterol supplementation.

I have shown that the LA/N-cp rat develops a high incidence of lesions by six months of age. The lesions do share many characteristics with lesions seen in man. The findings of this study indicate that the LA/N-cp rat has the potential to be an excellent model of atherosclerosis and deserves further evaluation. The possibility that the inciting lesion is the subendothelialization of damaged endothelial cells also requires more study.

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